Canadian Journal of Botany

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOLUME 30 NOVEMBER, 1952 NUMBER 6

STUDIES ON THE FORMATION OF VITAMIN C IN SLICES OF POTATO TISSUE[1]

BY E. A. M. ASSELBERGS AND F. J. FRANCIS[2]

Abstract

When slices of potato tissue are stored for two days at 23° C. in moist chambers or in aerated distilled water, the vitamin C content increases 100-300% as measured by the method of Roe *et al.* The increase does not occur in slices from freshly harvested potatoes. The physiological condition of the potatoes at the time of the experiment is important in determining the amount of increase in slices from potatoes stored for several months. Experiments with auxins and mannitol solutions show that there is no direct relationship between the process of water uptake and vitamin C formation. Solutions of indoleacetic acid and naphthaleneacetic acid increase the amount of vitamin formed. The results of experiments with potassium cyanide, sodium azide, malachite green, 8-hydroxyquinoline, sodium diethyldithiocarbamate and 2,4-dinitrophenol suggest that the formation of vitamin C is not directly related to any of the enzyme systems inhibited by the above chemicals. Infiltration of potato slices with solutions of fumaric and malic acid results in an increased formation of vitamin C as compared with the water controls.

Introduction

Potatoes contribute a large portion of the vitamin C intake of people in the low-income groups in Canada and the United States. In some countries with a low standard of living, potatoes form the chief source of vitamin C.

Freshly harvested potatoes contain approximately 30 mgm. of ascorbic acid per 100 gm. In two to three months, the content drops to 20-30% of the original value and remains at the "basic" level for the remainder of the storage period. Although many investigations are concerned with attempts to prevent this decrease in nutritional value, as yet, no practical methods have been successful.

Pett (10) found that tubers treated with ethylchlorohydrin showed an increased content of ascorbic acid (AA) after sprouting, particularly when the tubers were cut in half. Guthrie (5) confirmed Pett's work and found that the increase in ascorbic acid content would proceed without chlorohydrin treatment. Steward and Preston (19), working with thin slices of potato tissue in aerated distilled water or dilute salt solutions, also found an increase in ascorbic acid content.

[1] *Manuscript received for publication June 26, 1952.*

Contribution from the Department of Horticulture, Ontario Agricultural College, Guelph, Ont. Based on part of a thesis submitted by the first named author in partial fulfillment of the requirements for the degree of Master of Science in Agriculture. Supported in part by a grant from the Research Council of Ontario.

[2] *Lecturer.*

[The September number of this Journal (Can. J. Botany, 30 : 521-664. 1952) was issued September 30, 1952.]

Prokoshev, Dansheva, and Shamrai (12, 13, 14, 17) studied the conditions of storage for optimum development of vitamin C in potato slices and considered the increase in vitamin content to be of traumatic origin. The traumatic reactions of plant tissue are described as the complex of physiological and chemical processes caused by mechanical damage to the tissue and localized in the zone of injury. Development of the traumatic reactions is only possible where undamaged cells are in contact with the injured tissue. Squeezing of living tissue into a mash introduces a series of autolytic processes which have only a slight connection with the traumatic reactions. For instance, Mills (9) reported that the dehydroascorbic acid (DHA) content of potato slurry, prepared in a Waring Blendor, will be largely converted to 2,3-diketo-*l*-gulonic acid (DKA) in a few days. From experiments reported in the present paper, the breakdown of DHA in intact potato tissue does not show up as an increased DKA content.

The present work was attempted to investigate the enzyme systems responsible for the biosynthesis of vitamin C in slices of potato tissue.

Materials and Methods

Katahdin potatoes were grown locally and placed in storage at 3-4° C. Unless otherwise stated, the potatoes were brought to room temperature 7-10 days before use, to minimize the effect of temperature change and to lower the sugar content. The effect of varying concentration of ascorbic acid in different parts of the tuber was minimized by using only the center or pith of large (150 gm. or more) potatoes. In the course of each experiment, changes may be expected in both fresh and dry weight of the potato slices, and therefore all analyses are expressed on the basis of 100 gm. of original fresh weight.

The following procedure for preparing sterile potato slices was developed. After thorough cleaning in tapwater, a potato was peeled (a layer 1-2 cm. thick was removed), dipped in 95% alcohol, and flamed. The potato was then transferred to a canopy where "sterile" conditions were maintained with an ultraviolet lamp. A cylinder of potato tissue, cut perpendicular to the long axis of the potato, was removed with a glass borer. The cylinder was sliced by a device consisting of two Schick razor blades separated by a distance of 3 mm. This thickness was found by Shamrai (17) to be the optimum value for vitamin C formation. Steward (18) pointed out why this thickness is the optimum by studies on surface effects of potato slices.

The technique for storing potato slices in a warm humid atmosphere, as used by Shamrai (17), was abandoned because of the difficulty in preventing bacterial contamination. Two other methods were investigated. One of these, adapted from Steward (18), consisted of storing each slice in a test tube containing 20 ml. of autoclaved distilled water. Oxygen was bubbled through at the rate of 20-25 liters per hour. The apparatus was maintained at 33° C. in a storage cabinet. Another method consisted of storing the potato slices in large desiccators lined with wet filter paper. A Petri dish,

containing 50 ml. of 15% sodium hydroxide solution, was placed on the bottom of the desiccator to prevent a build-up of carbon dioxide. The opening in the desiccator lid was closed with a cotton plug. During the course of the experiment, the desiccators were stored in a temperature controlled cabinet.

It was more convenient and less expensive to store potato slices in desiccators than in test tubes. Therefore the former method was adopted in the chemical treatments. It was comparatively simple to infiltrate the potato slices with the desired solutions because of the uptake of liquid. In the infiltration procedure, four Whatman No. 3 filter papers were placed on the plate of the desiccator and moistened with 5 ml. of the appropriate solution. Two potato slices were placed on each filter paper and 1 ml. of solution was added to the top of each slice. During the course of the experiments, when the top surface of the slice appeared to be dry, another milliliter of solution was added.

The analyses for vitamin C (AA and DHA) were performed according to the method of Roe *et al.* (16), using a Klett-Summerson photoelectric colorimeter equipped with a 540 mμ filter. This method was chosen in order to obtain values for ascorbic acid, dehydroascorbic acid, and diketogulonic acid. It was thought that the additional data warranted the increased labor.

Results

Effect of Method of Storage

The effect of storing potato slices in aerated water and in moist desiccators is shown in Fig. 1. The increase in AA in slices from old potatoes is less in the former method. The lower amount of AA is not due to either a leaching

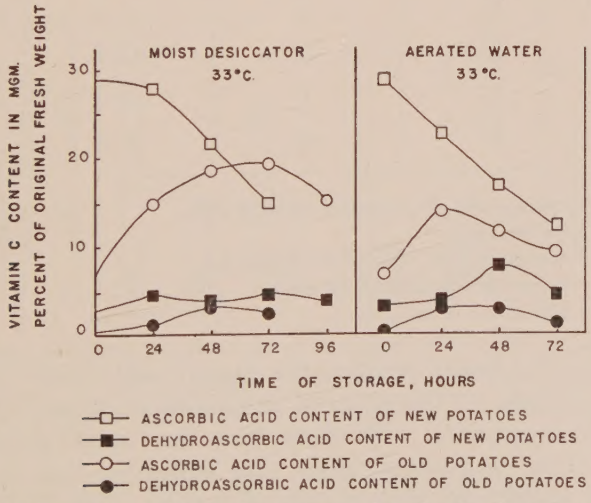


FIG. 1. Effect of time and condition of storage on the vitamin C content of potato slices.

effect of the water, because analyses of the solutions gave negative results, or to a shift from AA to DHA. In the moist desiccator storage method the AA increases for 72 hr. and then begins to decrease. The ratio of AA to DHA

as well as the total of the two decreases after 72 hr. The values for DKA content are not reported as they were less than 1 mgm per 100 gm. The difference in behavior of old and new potatoes is evident from Fig. 1.

Effect of Physiological Condition of Whole Potatoes

In preliminary work, it was noticed that considerable variation occurred in the increase in vitamin C content of the potato slices on storage in moist chambers. This variation is due, in part, to the physiological condition of the potato as shown by Table I. The data were taken from experiments on four potatoes which were chosen to represent the extremes of physiological condition. All potatoes had been stored for five months.

TABLE I

THE EFFECT OF PHYSIOLOGICAL CONDITION OF WHOLE POTATOES ON THE INCREASE IN FRESH WEIGHT AND VITAMIN C CONTENT OF POTATO SLICES IN MOIST DESICCATORS AT 23° C.

Description of potato	Increase in fresh weight in per cent	Time of storage, hr.	Vitamin C content[5] in mgm. % of original fresh weight	
			AA	DHA + DKA
Aged[1], cold[3]	34	0	10.0	2.4
	28			
	33	42	13.5	4.9
Aged, warm[4]	37	0	7.9	2.5
	34			
	30	42	19.5	4.8
Fresh[2], cold	47	0	9.0	3.7
	45			
	44	42	11.5	3.9
Fresh, warm	17	0	8.0	3.5
	15			
	18	42	16.5	2.9

[1] *Soft texture with irregular cork formation on thick skin.*
[2] *Firm texture with thin skin.*
[3] *Removed from storage (3-5° C.) three hours before use.*
[4] *Kept at room temperature 10 days before use.*
[5] *Average of the three slices in each series.*

The difference in texture between the "aged" and "fresh" potatoes was reflected in the weight of the slices of equal size from each potato at the beginning of the experiment. The slices from the "aged" potatoes weighed less than those from the fresh potatoes, thus indicating that the former had transpired more during the five months of storage. The water uptake, as evidenced by the change in weight, of the "aged" slices was intermediate between the warm and the "cold", and "fresh" slices. Apparently, the temperature treatment influences the water uptake in "fresh" slices much more than in the slices from "aged" potatoes. This effect may be related to

the starch–sugar equilibrium, as Hackett (7) has shown that the osmotic pressure of the cell fluid changes as water is absorbed.

The vitamin C content of the slices at the beginning of the experiment does not vary appreciably among the individual potatoes. The content after 48 hr. storage is influenced by temperature treatment and physiological age. The "aged" and "warm" slices gave the largest increase in vitamin C content. Values for DKA are not reported because they were less than 1 mgm. per 100 gm.

In the following experiments the variation due to physiological age (all potatoes were given the same temperature treatment) was minimized by using slices from the same potato as controls. In this way the results of various treatments within one experiment may be compared.

The Relationship Between Vitamin C Increase and Water Uptake

In preliminary experiments, designed to develop a method for storing potato slices for optimum development of vitamin C, it was observed that an increase in vitamin C content was usually accompanied by a corresponding increase in weight. This suggested that there might be a relationship between the two. To test this hypothesis, two experiments were set up. One experiment, with auxins, was designed to increase the water uptake and the other experiment with mannitol solutions, was designed to decrease the water uptake. The data are presented in Fig. 2 and Fig. 3 *A*.

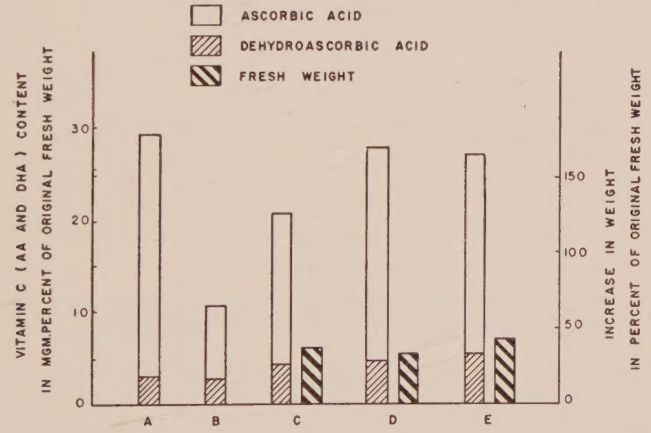


FIG. 2. Effect of infiltration with water (*C*), indoleacetic acid, 10 mgm./liter (*D*), and naphthaleneacetic acid, 10 mgm./liter (*E*) on the fresh weight and the vitamin C content of potato slices, stored in moist desiccators at 23° C. for 43 hr. (*A*) Vitamin C content of freshly harvested potatoes. (*B*) Vitamin C content of experimental potatoes at beginning of experiment.

The treatment with both indoleacetic acid and naphthaleneacetic acid increased the amount of vitamin C compared with the water controls. It was not established whether this increase was real or merely an increase in rate of formation. Infiltration with naphthaleneacetic acid increased the water uptake above that of the water controls but under the conditions of this

experiment the indoleacetic acid did not. Thus, there was no apparent relationship between the water uptake and the increase in vitamin C.

The mannitol solutions effectively prevented the uptake of water, and in the higher concentrations even decreased the water content. The formation of vitamin C proceeded as usual, again indicating that there is no direct relationship between the two.

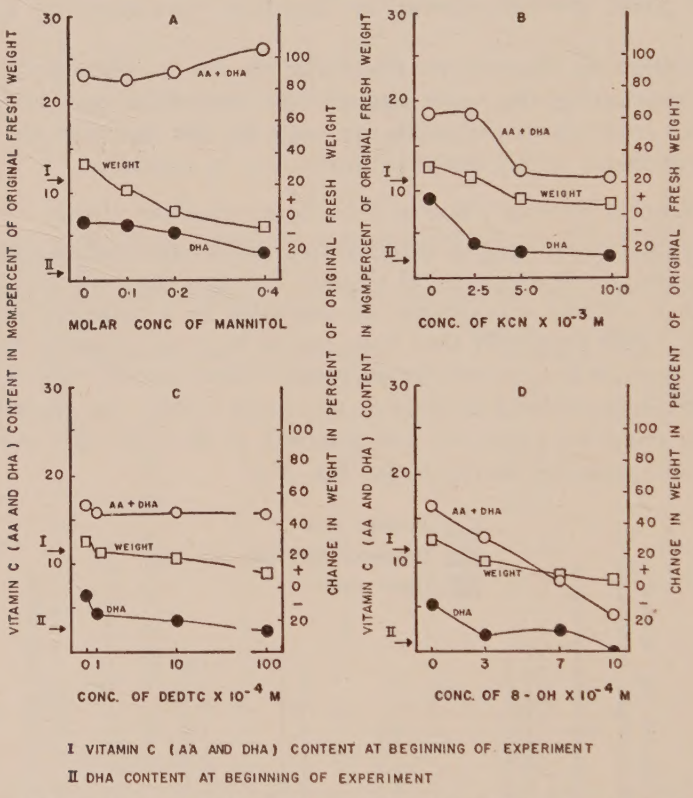


FIG. 3, A–D. Effect of various concentrations of chemical inhibitors on the fresh weight and the vitamin C content of potato slices stored in moist desiccators at 23° C. for 41 hr.

The possibility that the mannitol solutions interfered in the analyses for vitamin C was tested by analyzing a 0.4 *M* solution. There was no evidence of color formation.

Effect of Certain Enzyme Inhibitors

Potato slices were infiltrated with a series of enzyme inhibitors in the hope of relating the formation of vitamin C to a specific enzyme system. The inhibitors used, together with the system inhibited, were potassium cyanide (heavy metal enzymes), sodium diethyldithiocarbamate, and malachite green (copper containing enzymes) (11, 3), 8-hydroxyquinoline (iron and copper containing enzymes) (6), sodium azide (cytochrome oxidase) (8), and 2,4-dinitrophenol (phosphorylation mechanism) (2). The concentration of

the inhibitors were chosen to cover the range which blocked the enzyme system without obviously killing the tissue. The data are presented in Fig. 3 *B*, *C*, *D*, and Fig. 3 *E*, *F*, *G*.

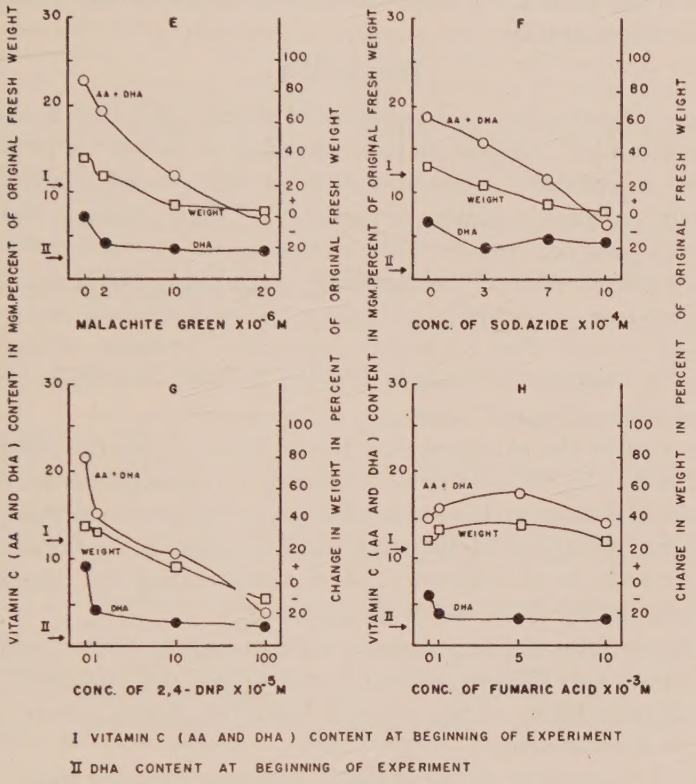


FIG. 3, E–H. Effect of various concentrations of chemical inhibitors and substrates on the fresh weight and the vitamin C content of potato slices stored in moist desiccators at 23° C. for 41 hr.

The action of the inhibitor, in every case except that with sodium diethyldithiocarbamate, reduced the formation of vitamin C. Malachite green and sodium diethyldithiocarbamate are both inhibitors of the enzymes containing copper but the formation of vitamin C is very sensitive to the action of the former and unaffected by the latter. The formation of vitamin C is more sensitive to the action of the inhibitor than is the process of water uptake.

The ratio of ascorbic acid to dehydroascorbic acid is increased by the action of mannitol and the chemical inhibitors. In each case the shift from ascorbic to dehydroascorbic acid is less than in that with the water controls. The values for diketogulonic acid content ranged from 1-2 mgm. per 100 gm. for the azide and hydroxyquinoline treatments. With 0.00001 *M* dinitrophenol solution, 3-4 mgm. of DKA appeared. The other treatments showed less than 1 mgm. of DKA per 100 gm. of fresh tissue. This amount is well within the experimental error of the method.

In Fig. 3 *H*, data are presented on the effect of infiltrating potato slices with solutions of fumaric acid. The formation of vitamin C is favored by the presence of the fumaric acid. A similar effect was observed with malic acid. The action of the substrate is more pronounced on the formation of vitamin C than on the water uptake.

Discussion

The general assumption that exposure of sliced vegetables to air causes a decrease in vitamin C content must be modified in the case of potatoes. On the contrary, the vitamin C content of sliced potatoes stored at high humidities at room temperature increases remarkably. This increase can only be obtained with potatoes after several months of previous storage during which time the vitamin C content has dropped considerably below that of freshly harvested potatoes. The vitamin C content from slices of old potatoes cannot be increased beyond that of freshly harvested potatoes. This raises the question as to whether the vitamin is being actually synthesized or is being regenerated from a "bound" form.

The question of the existence of a bound form of ascorbic acid has been postulated earlier by Reedman and McHenry (15) and supported or rejected by a number of workers. If the complex is a protein, as a number of investigators believe, the action of the auxins in increasing the vitamin content is more understandable, as evidence (1, 4) is accumulating that the amino acid metabolism is the site of action of the auxins.

The evidence from the experiments with mannitol, auxins, and chemical inhibitors shows that the processes of water uptake and vitamin C formation are not directly related. They may be indirectly related in so far as they can go on at the same time.

The actual penetration of the inhibitor solutions into the tissue is open to question. However, in the case of malachite green, dinitrophenol and hydroxyquinoline the tissue was actually stained to a depth of 1 mm. In these experiments no attempt was made to have a known amount of chemical taken up by the tissue. Rather it was felt that the concentration of the inhibiting solutions was the important factor

The interpretation of the results of the infiltration experiments is difficult because the enzyme inhibitors are not very specific. However, the fact that the vitamin C formation is blocked by a wide variety of inhibitors, each acting on a portion of the carbohydrate cycle, indicates that the process may be intimately related to the carbohydrate metabolism. It may be that the inhibitors reduce the energy available for vitamin C formation.

References

1. BONNER, J. Limiting factors and growth inhibitors in the growth of the *Avena* coleoptile. Am. J. Botany, 36 : 323-332. 1949.
2. BONNER, J. Relations of respiration and growth in the *Avena* coleoptile. Am. J. Botany, 36 : 429-436. 1949.
3. BOSWELL, J. G. Oxidation systems in the potato tuber. Ann. Botany, 9 : 55-76. 1945.

4. CHRISTIANSEN, G. S. and THIMANN, K. V. Studies on the growth and inhibition of isolated plant parts. VI. Nitrogen metabolism of *Pisum*. Am. J. Botany, 36 : 821-822. 1949.
5. GUTHRIE, J. D. Factors influencing the development of ascorbic acid and glutathione in potato tubers following treatment with ethylchlorohydrin. Contrib. Boyce Thompson Inst. 9 : 17-39. 1937.
6. GUZMAN BARRON, E. S., LINK, S. K. K., KLEIN, R. M., and MICHEL, B. E. The metabolism of potato slices. Arch. Biochem. 28 : 377-399. 1950.
7. HACKETT, D. P. The osmotic changes during auxin-induced water uptake by potato tissue. Plant Physiol. 27 : 279-285. 1952.
8. HACKETT, D. P. and THIMANN, K. V. The action of inhibitors on water uptake by potato tissue. Plant Physiol. 25 : 648-652. 1950.
9. MILLS, M. B., DAMRON, C. M., and ROE, J. H. Ascorbic acid, dehydroascorbic acid and diketogulonic acid. Anal. Chem. 21 : 707-709. 1949.
10. PETT, L. B. Changes in the ascorbic acid and glutathione content of stored and sprouting potatoes. Biochem. J. 30 : 1228-1232. 1936.
11. PONTING, J. D. and JOSLYN, M. A. Ascorbic acid oxidation and browning in apple tissue extracts. Arch. Biochem. 19 : 47-63. 1948.
12. PROKOSHEV, S. M. The traumatic reactions of potato tubers. Biokhymia, 8 : 124-132. 1942.
13. PROKOSHEV, S. M. Traumatic formation of vitamin C in potatoes. Biokhymia, 9 : 37-53. 1943.
14. PROKOSHEV, S. M. and DANSHEVA, E. Conditions of ascorbic acid biosynthesis. Biokhymia, 11 : 481-491. 1946.
15. REEDMAN, E. J. and MCHENRY, E. W. Combined ascorbic acid in plant tissue. Biochem. J. 32 : 85-93. 1938.
16. ROE, J. H., MILLS, M. B., OESTERLING, M. J., and DAMRON, C. M. The determination of diketo-*l*-gulonic acid, dehydroascorbic acid and *l*-ascorbic acid in the same tissue extract by the 2,4-dinitrophenylhydrazine method. J. Biol. Chem. 174 : 201-208. 1948.
17. SHAMRAI, E. I. The effect of sulphur dioxide in the biosynthesis of ascorbic acid in potato tubers. Biokhymia, 15 : 75-81. 1950.
18. STEWARD, F. C. The absorption and accumulation of solutes by living plant cells. IV. Surface effects with storage tissue. A quantitative interpretation with respect to respiration and salt absorption. Protoplasma, 17 : 436-453. 1932.
19. STEWARD, F. C. and PRESTON, C. Metabolic processes of potato discs under conditions conducive to salt accumulation. Plant Physiol. 15 : 23-61. 1940.

INHIBITION OF THE BACTERIOSTATIC ACTION OF LAURYLAMINE SACCHARINATE BY ORGANIC MATTER[1]

By P. H. H. Gray[2] and L. J. Taylor[3]

Abstract

Laurylamine saccharinate has been tested for its bacteriostatic powers against 30 species of bacteria, namely, 6 Gram-positive micrococci, 12 Gram-positive and 12 Gram-negative 'rods'. The compound was used in concentrations of 0.0005, 0.001, and 0.005% in plain broth, broth with glycerol, and broth with glycerol and skim milk, these adjuncts representing progressively increasing amounts of organic matter. The Gram-negative bacteria were the most resistant. The bacteriostatic action of the laurylamine was progressively reduced by the organic matter. *Staphylococcus aureus* was not killed by 0.005% laurylamine in the medium containing skim milk. Media containing phenol were used as a measure of comparison, and similar effects of organic matter were observed. Agar also reduced the bacteriostatic power of the laurylamine.

Laurylamine saccharinate has been suggested as a useful bactericidal compound in nasal medication (1). It was found to be bactericidal against *Staphylococcus aureus* at 0.005% concentration in peptone yeast-extract broth, and at weaker concentrations against more 'fastidious' bacteria, namely, *Streptococcus haemolyticus* and *S. viridans*, in infusion broths. Tests were also made with bacteria common in nasal secretions, namely, *B. coli*, strains of "*Pyocyaneus*", and "Friedlander's bacillus" (*Bacillus* of Friedländer, or *Pneumobacillus*, *Klebsiella pneumoniae*).

In view of the fact that the oral and nasal tracts are liable to receive a varied invasive flora, and are well supplied with organic debris, it was thought that a series of tests with this compound against as many species as possible should be made, with especial reference to the effects of organic matter.

Experimental

A 5% solution of the laurylamine* was pipetted aseptically into flasks of sterile media to give concentrations of 0.0005, 0.001, and 0.005%. From these medicated media, portions, 5 ml. of broth, or 10 ml. of agar, were pipetted into culture tubes. The tubed media were incubated for 24 hr. to test for sterility.

The following media were used:

Brain heart infusion broth (Bacto).....Medium I
Medium I with glycerol 1%.....Medium II
Nutrient broth (Bacto) with glycerol 1% and skim milk 5%....Medium III
Medium III with agar 1.5%.....Medium IV

[1] *Manuscript received December 7, 1951, and, as revised, July 28, 1952.*
Contribution from the Faculty of Agriculture, McGill University, Macdonald College, Que., Can. Macdonald College Journal Series No. 296.

[2] *Associate Professor and Chairman, Department of Agricultural Bacteriology, McGill University, Montreal, Que.*

[3] *Postgraduate student, Faculty of Graduate Studies and Research, McGill University, Montreal, Que.*

* *Delmar Chemicals Limited, Lachine, Que. provided the specimen of laurylamine saccharinate. A 5% solution was found to be self-sterilizing.*

TABLE I

EFFECTS OF LAURYLAMINE AND PHENOL IN CULTURES OF GRAM-POSITIVE MICROCOCCI, IN 48 HR.

Organism	Laurylamine, mgm./ml.	Medium				Phenol, mgm./ml.	Medium			
		I	II	III	IV		I	II	III	IV
Micrococcus cereus	0.005	++	++	++	++	2.0	−	−	+	++
	0.01	−	−	++	++	3.0	−	−	−	+
	0.05	−	−	−	+	4.0	−	−	−	−
Micrococcus perflavus	0.005	++	++	++	++	2.0	−	+	++	++
	0.01	−	+	++	++	3.0	−	+	+	+
	0.05	−	+	+	+	4.0	−	−	−	+
Micrococcus roseus	0.005	−	−	++	++	2.0	−	−	+	++
	0.01	−	−	++	++	3.0	−	−	−	+
	0.05	−	−	+	+	4.0	−	−	−	−
Gaffkya tardissima	0.005	++	++	++	++	2.0	−	++	++	++
	0.01	−	−	+	+	3.0	−	+	++	++
	0.05	−	−	−	+	4.0	−	−	+	++
Staphylococcus aureus	0.005	++	++	++	++	2.0	+	++	++	++
	0.01	−	++	++	++	3.0	−	+	+	+
	0.05	−	−	+	++	4.0	−	−	+	+
Staphylococcus cereus	0.005	−	−	++	++	2.0	−	+	++	++
	0.01	−	−	+	+	3.0	−	−	+	+
	0.05	−	−	+	+	4.0	−	−	−	−

NOTE: *In Tables I-IV the − sign indicates no visible growth, + indicates growth, ++ vigorous growth.*

TABLE II

EFFECTS OF LAURYLAMINE AND PHENOL IN CULTURES OF GRAM-POSITIVE RODS, IN 48 HR.

Organism	Laurylamine, mgm./ml.	Medium				Phenol, mgm./ml.	Medium			
		I	II	III	IV		I	II	III	IV
Bacillus cereus	0.005	++	++	++	++	2.0	+	+	++	++
	0.01	−	−	++	++	3.0	−	−	++	++
	0.05	−	−	−	+	4.0	−	−	+	+
Bacillus mesentericus	0.005	++	++	++		2.0	+	+	+	+
	0.01	++	++	++		3.0	−	−	+	+
	0.05	−	−	++		4.0	−	−	−	+
Bacillus ruminatus	0.005	+	++	++	++	2.0	+	+	+	++
	0.01	−	−	++	++	3.0	−	+	+	+
	0.05	−	−	−	+	4.0	−	−	−	+
Corynebacterium diphtheriae	0.005	−	++	++	++	2.0	++	++	++	++
	0.01	−	−	++	++	3.0	+	+	+	+
	0.05	−	−	−	−	4.0	−	−	+	+
Corynebacterium hoffmani	0.005	−	+	++	++	2.0	+	+	++	++
	0.01	−	−	++	++	3.0	−	+	+	+
	0.05	−	−	+	+	4.0	−	−	+	+
Corynebacterium xerosis	0.005	++	++	++	++	2.0	++	++	++	++
	0.01	−	−	++	++	3.0	+	+	++	++
	0.05	−	−	−	−	4.0	−	−	+	+

TABLE II—(*Concluded*)

EFFECTS OF LAURYLAMINE AND PHENOL IN CULTURES OF GRAM-POSITIVE RODS, IN 48 HR.—(*Concluded*)

Organism	Laurylamine, mgm./ml.	Medium				Phenol, mgm./ml.	Medium			
		I	II	III	IV		I	II	III	IV
Proactinomyces (*Nocardia*) *erythropolis*	0.005	++	++	++	++	2.0	−	+	+	+
	0.01	−	−	+	++	3.0	−	−	+	++
	0.05	−	−	−	−	4.0	−	−	−	−
Proactinomyces (*Nocardia*) sp.	0.005	−	−	++	++	2.0	+	+	+	+
	0.01	−	−	−	−	3.0	−	−	+	++
	0.05	−	−	−	−	4.0	−	−	−	−
Mycobacterium berolinensis	0.005	+	++	++	++	2.0	−	+	+	++
	0.01	−	++	++	++	3.0	−	−	+	++
	0.05	−	−	+	+	4.0	−	−	−	−
Mycobacterium phlei (M)	0.005	−	−	+	++	2.0	−	+	++	++
	0.01	−	−	−	−	3.0	−	+	+	++
	0.05	−	−	−	−	4.0	−	−	+	+
Mycobacterium phlei (R)	0.005	++	++			2.0	+	+	++	++
	0.01	−	++			3.0	−	−	+	+
	0.05	−	++			4.0	−	−	−	−
Mycobacterium stercusis	0.005	−	++	++	++	2.0	+	+	++	++
	0.01	−	−	+	+	3.0	−	−	+	+
	0.05	−	−	+	+	4.0	−	−	−	−

TABLE III

EFFECTS OF LAURYLAMINE AND PHENOL ON GRAM-NEGATIVE BACTERIA (*Bacterium* GROUP), IN 48 HR.

Organism	Laurylamine, mgm./ml.	Medium				Phenol, mgm./ml.	Medium			
		I	II	III	IV		I	II	III	IV
cloacae	0.005	++	++	++		2.0	+	+	++	++
	0.01	+	++	++		3.0	−	−	++	++
	0.05	−	+	++		4.0	−	−	+	+
coli	0.005	++	++	++	++	2.0	+	++	++	++
	0.01	−	+	++	++	3.0	−	−	+	+
	0.05	−	−	−	+	4.0	−	−	−	−
enteritidis	0.005	++	++	++		2.0	+	+	++	++
	0.01	++	++	++		3.0	−	−	+	+
	0.05	−	+	++		4.0	−	−	−	−
iophagum	0.005	++	++			2.0	−	+	+	+
	0.01	++	++			3.0	−	−	+	+
	0.05	−	++			4.0	−	−	−	−
paratyphi	0.005	++	++	++		2.0	−	+	++	++
	0.01	++	++	++		3.0	−	+	+	+
	0.05	+	+	++		4.0	−	−	+	+
pneumoniae	0.005	++	++			2.0	+	++	++	++
	0.01	++	++			3.0	−	−	++	++
	0.05	−	++			4.0	−	−	+	+

TABLE IV

EFFECTS OF LAURYLAMINE AND PHENOL ON GRAM-NEGATIVE BACTERIA (*Pseudomonas* GROUP)

Organism	Laurylamine, mgm./ml.	Medium				Phenol, mgm./ml.	Medium			
		I	II	III	IV		I	II	III	IV
aeruginosa	0.005	++				2.0	+	+	++	++
	0.01	++				3.0	−	−	+	+
	0.05	++				4.0	−	−	−	−
indoloxidans	0.005	++	++			2.0	−	+	+	+
	0.01	++	++			3.0	−	−	−	+
	0.05	−	++			4.0	−	−	−	−
phaseoli	0.005	−	−	++	++	2.0	−	+	+	++
	0.01	−	−	++	++	3.0	−	−	+	+
	0.05	−	−	−	+	4.0	−	−	−	−
pictorum	0.005	+	++	++	++	2.0	−	−	+	++
	0.01	−	−	++	++	3.0	−	−	−	+
	0.05	−	−	+	+	4.0	−	−	−	−
syncyanea	0.005	++				2.0	−	+	++	++
	0.01	++				3.0	−	+	+	++
	0.05	++				4.0	−	−	+	+
tumefaciens	0.005	−	++	++	++	2.0	−	−	+	++
	0.01	−	−	+	++	3.0	−	−	−	+
	0.05	−	−	−	+	4.0	−	−	−	−

The media thus represented material with progressively increasing amounts of organic matter.

As a measure of comparison the same media were used under the same conditions with phenol, in "equivalent" bacteriostatic concentrations; preliminary tests showed that 0.3% phenol in broth was bacteriostatic for some of the common bacteria to be tested; the concentrations used in media I-IV were 0.1, 0.2, 0.3, and 0.4%. The results for the lowest concentration have been omitted from the tables.

The medication media were inoculated by loop from well-shaken cultures of the bacteria grown for 24 hr. in suitable broths, usually nutrient broth, but with glycerol provided for the mycobacteria. The bacteria used were some of those in the pure culture collection at Macdonald College, as shown in tables of results (Tables I-IV). Before use, each culture was examined for morphological purity*. The medication cultures were incubated at 30° C. and examined after 24, 36, and 48 hr. Relative growth was determined by cloudiness in the broth media, and by the diameters of colonies on the agar.

Results

Reference to Tables I-IV shows that the addition of organic matter to the brain heart infusion broth and the nutrient broth reduced the bacteriostatic power of both the laurylamine and the phenol against most of these bacteria. It should be noted that *Staphylococcus aureus* grew in the broth containing laurylamine 0.005% (0.05 mgm. per ml.) in the presence of skim milk (Medium III).

In order to simplify the tabulated results an analysis is presented in Table V, in which the 'index of bacteriostasis' for each group of microorganisms is shown.

The index is derived by dividing the number of cultures in each group into the summation of − signs under each concentration; complete bacteriostasis

TABLE V

'INDICES OF BACTERIOSTASIS' IN CULTURES WITH LAURYLAMINE

Bacterial group	Laurylamine, % (phenol, %)		
	0.0005 (0.2)	0.001 (0.3)	0.005 (0.4)
6 Gram-positive micrococci	0.7 (1.1)	1.7 (1.0)	2.2 (3.0)
12 Gram-positive rods	0.6 (0.3)	2.0 (1.4)	3.0 (3.0)
6 *Bacterium*	0.0 (0.2)	0.2 (1.8)	1.1 (2.8)
6 *Pseudomonas*	0.5 (1.2)	1.0 (2.3)	1.5 (3.7)

* *The culture named Bacterium pneumoniae was, at the time of its accession some years ago, named Klebsiella pneumoniae by its donor; it is, however, a motile microorganism.*

against all of the cultures in a group would be represented by the value four; that is, the higher the index value the more effective is the disinfectant. This arrangement is purely arbitrary, as there is no reason to assume that the differentiating characters of the bacteria in each group bear any relation to their physiology. Nevertheless, it should be noted that the Gram-negative bacteria are the most resistant. The indices of bacteriostasis for phenol (shown in parentheses) appear to be somewhat similar to those of laurylamine for all groups, but suggest that the concentration of 0.4% is equally bacteriostatic for all groups.

Table VI shows the "inhibition indices", derived in a similar manner but by summating the number of − signs under each column headed Medium I, II, III, and IV; the total number of − signs divided into the + signs in each column gives the index of inhibition for each medium, i.e., demonstrates the effectiveness of the added organic matter in reducing inhibition.

The strains of the *Bacterium* group are clearly the most able to develop in the presence of less organic matter than are any of the others, the index for Medium II being 17 (maximum for that group, 18). The *Pseudomonas* group required the further addition of skim milk, the index being eight for Medium III and 18 (maximum) for Medium IV; the addition of agar was thus required to produce maximum inhibition. The effects of organic matter reducing bacteriostasis by phenol (0.2, 0.3, and 0.4%) is also shown in Table VI; maximum inhibition was not reached in any of the groups.

TABLE VI

'INDICES OF INHIBITION' OF BACTERIOSTASIS, BY ORGANIC MATTER IN CULTURES WITH LAURYLAMINE AND PHENOL

Bacterial group	Compound	Medium			
		I	II	III	IV
Gram-positive micrococci (6)	Laurylamine	0.29	0.63	8.0	18.0
	Phenol	0.06	0.63	2.0	5.0
Gram-positive rods (12)	Laurylamine	0.3	0.64	3.0	4.1
	Phenol	0.4	0.89	4.1	6.2
Bacterium group (6)	Laurylamine	2.0	17.0	17.0	18.0
	Phenol	0.3	0.6	5.0	5.0
Pseudomonas (6)	Laurylamine	1.0	1.6	8.0	18.0
	Phenol	0.06	0.4	1.3	2.6

This work is being continued to ascertain the bactericidal action of laurylamine against several of the bacteria named above, and others freshly isolated from the oral and nasal passages.

Reference

1. HAMILTON, W. F. and TURNBULL, F. M. J. Am. Pharm. Assoc. 39 : 378-382. 1950.

OBSERVATIONS ON THE HOST RANGE OF *CUSCUTA EUROPEA* AMONG THE COMPOSITAE[1]

ERIKA E. GAERTNER[2]

Abstract

One hundred and twenty-eight species of 44 genera of the family Compositae were examined as to their susceptibility to the dodder, *Cuscuta europea*. The indices presented are thus to supply additional data about host preference in the genus *Cuscuta*.

A chance visit to the Bergianus Botanical Gardens in Stockholm while the writer was attending the Seventh Botanical Congress disclosed a dodder infection of such a nature that it should contribute to our general concept of the host range of this dodder. The report on this infection should be considered as a supplement to the previous papers of the writer (1, 2) on the host range of *Cuscuta* spp. Most of the plants here listed were never before investigated as to their relationship with dodder. Because of the use of dodder as a vector by the students of virus diseases, additional data appear to be of immediate value.

The dodder, later identified as *C. europea* L., was distributed throughout the planting of Compositae in such a fashion that the information obtained appears to be as valuable as that of the experiments mentioned above. The plot contained 44 genera with 128 species of which 12 genera with 45 species were parasitized. There were a few additional genera and species in the plot but, because of their poor germination and growth, no record of them was taken.

Each species of the potential host was grown in a small plot of approximately 4 sq. ft. These plots were about one foot apart. The dodder would often be absent from a plot adjoining some heavily infested ones. Where the same species was found twice, regardless of position in the plantation, it was consistent with respect to dodder infection.

It was not possible to trace the history of these plots, except that the original Compositae planting, which contained many perennials was well established. The plants susceptible to *C. europea* and those thought, by circumstantial evidence, not to be susceptible to the said dodder, are listed below in a Supplementary Host Index and a List of Plants Found Growing Free of *C. europea*.

[1] *Manuscript received May 14, 1952.*

Presented before the General Section, Bot. Soc. Am. Meetings, Minneapolis, Minn. Sept. 12, 1951.

[2] *Sessional lecturer, Department of Botany, McMaster University, Hamilton, Ont.*

* *The Index Kewensis, all parts, as well as the eighth edition of Gray's Manual, were used to check on the plant names as found on the labels in the garden. When the labels did not give an authority and either several authorities or none were found, quotation was impossible.*

Supplementary Host Index of *Cuscuta europea* L.

Achillea compacta Lam.
A. magna L.
A. odorata L.
A. ptarmica L.
Artemisia absinthium L.
A. arbotanum L.
A. glauca Pall.
Aster amellus L.
A. acer L.
A. divaricatus L.
†*A. corymbosus* Ait.
A. grandiflorus L.
A. herveyi A. Gray
A. incissus Fisch.
A. laevis L.
A. macrophyllus L.
A. novae-angliae L.
A. novae-belgii L.
A. puniceus L.
A. sagitifolius Wedem.
A. sikkinnensis Hook. E.C. and Thoms.
A. tradescantii L.
Cacalia suaveolens L., Synosma suaveolens Raph.
Chrysanthemum anserinifolium Bois.
C. balsamita L., Balsamita vulgaris Will.
C. vulgare Bernth.
Erigeron annuus (L.) Pers.
E. divergens U.K.
E. glabellus Nutt.
Helianthella uniflora Torr. and Gray
Inula brittanica
I. euvifolia L.
I. germanica L.
I. helenium L.
I. oculus christi L.
I. sabuletorum
I. salicina L.
I. salicina var. *minor*
Senecio pseudo-arnica Less.
Solidaster luteus L. Green (Solidago spec. × Aster ptarmicoides Torr. and Gray)
Solidago canadensis L., *S. flexicaulis* L., S. latifolia L.
S. gigantea Ait. v. *leiophylla* Fern., S. serotina Ait.
S. rigida L.
S. shortii Torr. and Gray
S. ulmifolia Muhl.
S. virgaurea L.
S. sp.
Tanacetum vulgare f. *crispum*

List of Plants Found Growing Free of *Cuscuta europea* L.

Achillea cartillaginea Led.
A. millefolium L., †A. collina Beck.
A. setacea Waldst.
A. tomentosa L.
Ageratum houstonianum Mill.
Ambrosia artemisiifolia L.
A. trifida L.
Anacyclis pyrethrum DC.
A. radiatus Loisel.
Anaphalis margaritacea (L.) Benth. and Hook.
Anthemis altissima L.
A. austriaca Jack.
A. carpathica Willd.
Aster sedifolius L., A. acris L.
A. tongolensis Franch.

† *Synonym under which species was labeled.*

List of Plants Found Growing Free of *Cuscuta europea* L.—*Concluded*

Asteromea mongolica Kitamura
A. linosyris Bernh.
A. multiflorus Ait.
Bidens feraefolia DC.
B. infirma Ferr.
B. laevis (L.) BS
B. villosa L.
Brachycome iberidifolia Benth.
Carpesium eximium C. Winkl.
Cenia turbinata Pers.
Chrysanthemum carinatum Schousb.
C. coronarium L.
C. corymbosum L.
C. frutescens L.
C. lacustre Brot.
C. maximum Ram.
C. myconis L.
C. parthenoides v. *nana* hort. Voss.
C. serotinum
C. virido-hirtum Thell.
Cilfume perfoliatum L.
Coreopsis bigelowii Voss.
C. lanceolata L.
C. pubescens Ell. f.
C. stilmanii Blake
Egletes viscosa Less.
Eupatorium cannabinum L.
E. rugosum Houtt., E. urticifolium Reichard
Felicia fragilis Cass.
Grindelia integrifolia DC.
Guizotia abyssinica (L.f.) Cass.
Helianthus annuus L.
H. debilis Nutt.
Helichrysum arenarium Moench.
H. bracteatum Andr.
H. foetidum Moench.
Heliopsis helianthoides (L.) Sweet
Helipterium manglesii F. Muell.
H. roseum Benth.

Summary

In the Supplementary Host Index here presented three genera of Compositae (*Cacalia*, *Helianthella*, and *Solidaster*) are listed for the first time as host plants for any species of *Cuscuta*, five genera as new hosts to *C. europea* (*Cacalia*, *Helianthella*, *Inula*, *Senecio*, and *Solidaster*). All but five of the species listed in the Supplementary Host Index were not previously reported to be parasitized by *C. europea*. Krohn (3) reported two of them (*Inula salicina* and *Tanacetum vulgare*) as not becoming parasitized. Of the plants observed not to be affected by the dodder, *Achillea millefolium* has been previously found susceptible to *C. europea*. Four other not infected plants (*Helianthus annuus*, *Ambrosia artemisiifolia*, *A. trifida*, and *Eupatorium rugosum*) were previously found susceptible to another dodder species. Of the 83 species of plants not affected by *C. europea*, only two have been previously considered as potential hosts. In these two indices, 16 new genera were added to the list of plants examined in their relationship to *Cuscuta* spp.

References

1. Gaertner, Erika E. Some studies in the biology of *Cuscuta gronovii*. Thesis for degree of M.S., Cornell Univ. 1945. (Unpublished.)
2. Gaertner, Erika E. Studies of seed germination, seed identification, and host relationships in dodders, *Cuscuta* spp. Cornell Univ. Agr. Expt. Sta. Mem. 294. 1950.
3. Krohn, V. Kurzer Bericht uber *Cuscuta halophyta* Fries. Phytopath. Z. 7 : 505–514. 1934.

THE PROTEOLYTIC ENZYMES OF MICROORGANISMS

IV. PARTIAL PURIFICATION AND SOME PROPERTIES OF EXTRACELLULAR PROTEASE FROM *MORTIERELLA RENISPORA* DIXON-STEWART[1]

By L. R. Wetter

Abstract

A protease concentrate was obtained from the culture medium of *Mortierella renispora* Dixon-Stewart (PRL 26) by repeated precipitation with ammonium sulphate. The specific activity of the mold protease compared favorably with that of crystalline trypsin. The pH optimum was broad, with a maximum at a pH of 7.5 when hemoglobin was used as the substrate. A study of the pH stability characteristics showed that it was stable over a wide range (4.9 to 9.5) at 1° C. and 25° C. Ferrous ions caused a considerable increase in the activity of the enzyme preparations, other metals were ineffective as activators.

Introduction

Most of the work on the proteolytic enzymes of molds has employed extracts of the mycelium rather than the medium. Johnson (8) and Berger (3) have detected one proteinase and at least five peptidases in the mycelium of different molds. Ito (7) has reported the presence of proteolytic activity in the mycelium of *Penicillium notatum*. By employing a combination of acetone precipitation and ultrafiltration, Johnson and Peterson (8) obtained an appreciable purification of proteolytic enzyme from the mycelium of *Aspergillus parasiticus*.

In 1950, Crewther and Lennox (5) reported the isolation of crystals containing protease activity from the culture medium of *Aspergillus oryzae*. Dion (6) tested the culture medium of 289 fungi and actinomycetes, and found that 20 produced high yields of proteolytic activity. This communication describes the isolation and purification of extracellular proteolytic enzymes produced by a member of the phycomycetes, *Mortierella renispora* Dixon-Stewart (PRL 26). Some properties of the preparation were determined.

Experimental

Methods

The following procedure, based on Anson's method for trypsin (2) was used to determine proteolytic activity. A substrate solution of denatured hemoglobin was prepared from dried hemoglobin powder (Eimer and Amend) and stored in waxed paper cartons at −30° C. One milliliter of protease solution was incubated with 5 ml. of hemoglobin substrate at 25° C. After 10 min., the digestion was terminated by the addition of 5% trichloroacetic acid and the precipitate removed by filtration. Five milliliters of filtrate were mixed with 10 ml. of 0.5 *N* sodium hydroxide, and then 3 ml. of phenol reagent was added. Ten minutes later the blue color was read as per cent transmission

[1] *Manuscript received June 17, 1952.*

Contribution from the National Research Council, Prairie Regional Laboratory, Saskatoon, Saskatchewan. Issued as Paper No. 142 on the Uses of Plant Products and as N.R.C. No. 2830.

at 650 mμ in a Model B Beckman spectrophotometer. Blanks were obtained by adding 1 ml. of enzyme solution to a trichloroacetic acid – hemoglobin mixture.

Since metals were found to interfere with the Anson method as described above, the following modification was employed. The optical density of the filtrate was determined at 280 mμ. The increase in optical density was converted to enzyme units as described by Northrop (12, p. 305).

One unit of enzyme was defined as the amount which digested hemoglobin under the standard conditions at an initial rate such that there was liberated per minute an amount of split products not precipitated by trichloroacetic acid which gives the same color with phenol reagent as one milliequivalent of tyrosine (12). The method for determining the proteolytic unit and constructing the curve relating activity units to color value was that used by Anson (2).

Reducing sugars were determined by the colorimetric modification of the Somogyi method (10). Nitrogens were determined by the micro-Kjeldahl method. Since trichloroacetic acid failed to precipitate the protein in the culture medium, protein nitrogen was estimated by precipitating it from solution with equal portions of 15% tannic acid and estimating the nitrogen in the insoluble material. The mixture was allowed to stand at room temperature overnight before washing the precipitate twice with 7.5% tannic acid. Experiments on thoroughly dialyzed enzyme solutions, showed that tannic acid precipitated all the protein present.

Materials

The proteolytic enzymes were produced in 30 liter stainless steel fermentors, similar in construction to those described by Rivett *et al.* (13). Fifteen liters of medium consisting of 2% Klim, 2% glucose, 0.1% monobasic potassium phosphate, and tap water were sterilized in each fermentor for one hour. In some cases the glucose and Klim were sterilized separately to reduce the amount of browning in the medium. Each fermentor was inoculated with 600 ml. of a three-day-old culture grown in shake flasks on the above medium. The fermentations were agitated at approximately 160 r.p.m. and aerated at 0.5 liters of sterile air per liter of medium per minute. Fermentation took place at 30° C. until the proteolytic activity was sufficiently high (approximately 60 hr.). The mycelium was separated from the medium by filtration and the clear yellow liquid, containing the proteolytic enzymes, was stored under toluene at 1° C.

Results and Discussion

Concentration of the Protease

When the culture medium was concentrated approximately 15 times at 40° C. or lower by vacuum distillation in a "Precision Laboratory" evaporator very little loss in activity occurred. Since activity losses were appreciable

above 40° C., the apparatus was operated with distilled water until equilibrium at the desired temperature was attained. The low loss in activity is shown by the data listed in Table I.

TABLE I

PERCENTAGE RECOVERY OF PROTEOLYTIC ACTIVITY AFTER EVAPORATION AT 40° C.

Preparations	Per cent activity recovered
1	99
2	89
3	95
4	100
5	97

The culture medium was found to contain from 1% to 2% reducing sugar when the fermentation was terminated at the time corresponding to maximum protease production. The glucose can be removed either by absorption with yeast or by precipitation of the proteolytic activity with ammonium sulphate. Although the first method removed the glucose quantitatively, it was abandoned because of the possibility of contamination by yeast proteins.

The fractionation consisted of repeated precipitations of the proteolytic activity with ammonium sulphate. Precipitation of the protease was obtained by slowly adding solid ammonium sulphate with constant stirring to a final concentration of 3.25 *M*. The mixture was adjusted to pH 6 with 1 *N* sodium hydroxide and stored at 10° C. for two or three days. The precipitate was removed by centrifugation and extracted with approximately one-fifth the original volume of distilled water. The sample was again centrifuged and the precipitate discarded. The supernatant was designated as Sample *A*. Reprecipitation of Sample *A* with ammonium sulphate as described resulted in Sample *B*. Sample *C* was obtained in a similar manner from *B* (See Table II).

TABLE II

THE EFFECT OF AMMONIUM SULPHATE FRACTIONATION OF VARIOUS COMPONENTS IN A MOLD PROTEASE CONCENTRATE (PRL 26)

Fractions*	Total activity (units)	Total** nitrogen (mgm.)	Specific activity (units/mgm. *N*)	Reducing sugars (gm.)
Concentrate	11.32	390	0.029	117
Sample *A*	10.78	145	0.074	4
Sample *B*	8.33	100	0.083	Trace
Sample *C*	7.61	87	0.088	Trace

* *See text for designation of Samples A, B, C.*
** *Tannic acid precipitated nitrogen.*

The results for a typical concentration experiment are given in Table II. The first precipitation (A) removed approximately 97% of the reducing sugars and an additional precipitation removed all but traces of the sugars. In Sample *A* the loss of proteolytic activity was small, while the decrease in total nitrogen was considerable with the result that a 2.5-fold increase in specific activity was obtained. Although there was a threefold increase in activity after three precipitations, for gross concentration of the protease one precipitation was sufficient. Attempts to crystallize the protease of PRL 26, after three precipitations, by the method of Crewther and Lennox (5) as well as by several other methods failed.

Reports in the literature (12, p. 137) give the value of 0.16 for the specific activity of five times crystallized trypsin. Since recrystallized trypsin prepared in this laboratory yielded a specific activity of 0.08 and samples of protease prepared from PRL 26 gave activities of approximately 0.085, it appears that the activity of the new protease preparation compares favorably with recrystallized trypsin. It is noteworthy, however, that Crewther and Lennox (5) reported a crystalline preparation of protease from *Aspergillus oryzeae* to be 100 times more active than trypsin when hemoglobin was used as the substrate.

pH Optimum of PRL 26 Protease

McConnell (9) has shown that the pH optimum for the crude medium was approximately 7.5 when gelatin was used as the substrate. In the present

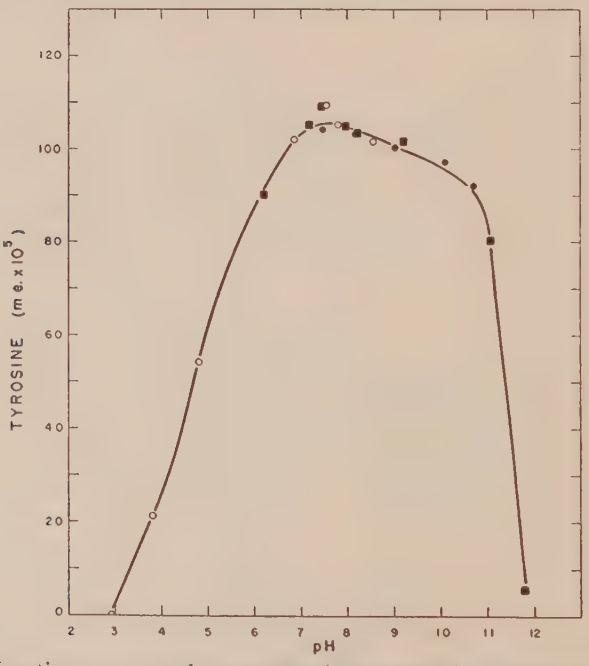


FIG. 1. pH optimum curve of concentrated protease using hemoglobin as substrate. The three sets of points correspond to determinations done at different times on the same enzyme preparation.

investigation hemoglobin was used as the substrate and a partially purified enzyme preparation comparable to Sample *C* was used. Varying amounts of acid or base were added to the hemoglobin to obtain the pH range. The pH recorded in Fig. 1 is that measured by using the glass electrode on samples after the 10 min. incubation period.

Fig. 1 compares the rate of hydrolysis with the change in pH. The optimum pH for hydrolysis is approximately 7.5 which agrees well with McConnell's (9) reported value for the crude enzyme. An interesting point is the fact that the optimum is quite broad, ranging from pH 6.5 to 10.5. The rate of hydrolysis falls off rapidly both for more acid and more alkaline conditions. This decrease is particularly noticeable on the basic side of the optimum where the tyrosine released drops from 90 me. to 6 me. in approximately one pH unit (10.8 to 11.8).

The pH optimum for the mold enzyme is similar to that of trypsin (pH 8–9). It is interesting that the optimum for the two substrates (hemoglobin and gelatin (9)) agree so closely. Northrop (11) reported that pH optimums are often dependent on substrate, as the rate of hydrolysis is related to the ionized protein (substrate) present.

Effect of pH and Temperature on the Stability of the Enzyme

During the course of the present investigation it was noticed that certain enzyme preparations lost much of their activity when stored for several days in acidic solutions. The marked decrease in the rate of hydrolysis on the acid and alkaline side of the pH optimum suggested that it would be of interest to investigate the pH stability of the enzyme. The experiment was designed in such a way that enzyme solutions were added to solutions of various buffers ranging in pH from 2.9 to 10.9. A previous experiment indicated that the salts used for preparing the buffers had no effect on the activity of the enzyme. The 1° C. samples were kept in an ice-water bath in the cold room and aliquots were withdrawn to determine the amount of enzyme present after varying intervals of time. Samples maintained at 25° C. were treated in the same way.

The results given in Figs. 2 and 3 show that the enzyme is stable between pH 4.9 and 9.5 over a considerable period of time for the temperatures investigated. The loss in activity becomes apparent below pH 4 especially when stored at 25° C. When the mold protease is stored at 1° C. no appreciable loss occurs until the hydrogen ion concentration is quite high (pH = 2.9). At similar acid concentrations samples held at 25° C. have been almost completely destroyed. A loss of 65% of the enzyme is observed after two hours at pH 2.9 and 25° C. A definite destruction of the enzyme is exhibited at pH 10.9 when it is stored at 25° C.; the loss at 1° C. is negligible.

A comparison of the pH-optimum curve (Fig. 1) and the pH-stability curves (Figs. 2 and 3) was of interest. The loss of activity and the decrease in digestion at alkaline pH's took place at approximately the same point (pH 10). However, the enzyme was stable in solutions having a pH of 4.9 while the rate

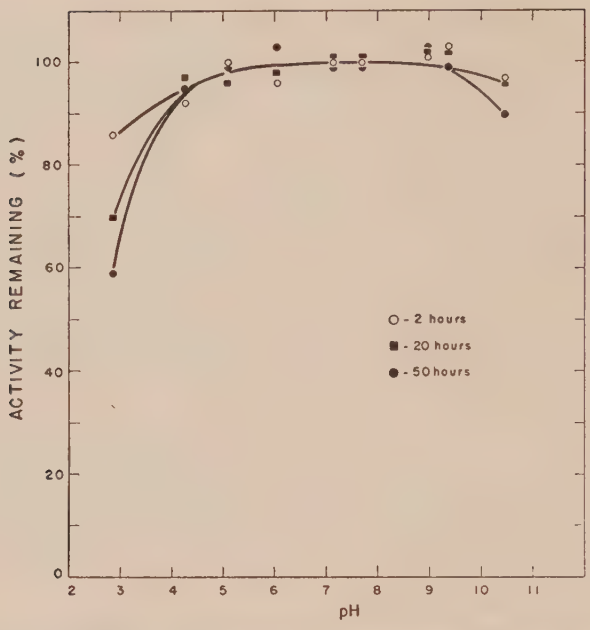


FIG. 2. The effect of pH on the stability of the protease (PRL 26) kept at 1° C. for varying lengths of time.
o = 2 hr.; ▪ = 20 hr.; • = 50 hr.

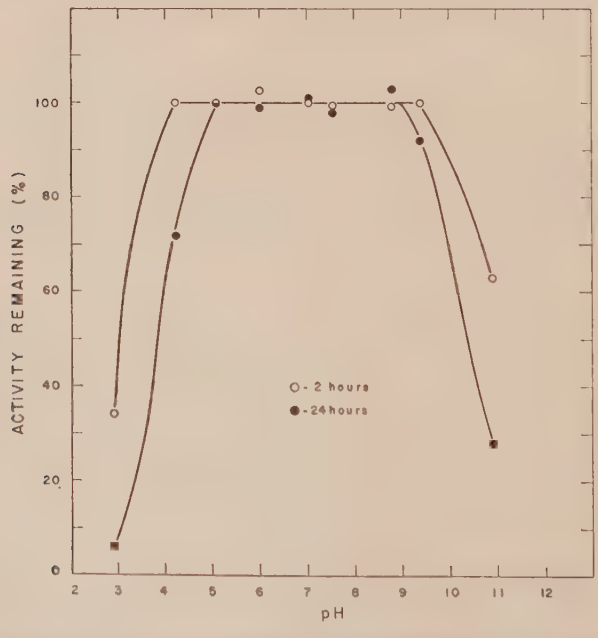


FIG. 3. The effect of pH on the stability of the protease (PRL 26) kept at 25° C. for varying lengths of time.
o = 2 hr.; • = 24 hr.

of hydrolysis decreased rapidly below pH 6.9. This would suggest that the decrease in hydrolysis rate with increase in acidity was not due to destruction but rather to a change in the ionization of the enzyme and/or the substrate.

Effect of Activators on the Enzyme

Snoke and Neurath (15) have reported that ferrous, magnesium, and manganese ions bring about an appreciable increase of the proteolytic activity in striated rabbit muscle. Adams and Smith (1) found that the addition of ferrous ions resulted in an increase in the activity of one of the two pituitary proteinases they were studying. An increase in the activity of trypsin by nickel ions has been reported by Sagai (14). The apparent activation of trypsin by metals, such as chromium, magnesium, nickel, and cobalt ions does not appear to be definitely established (4, 14).

TABLE III

INFLUENCE OF VARIOUS SUBSTANCES ON THE ACTIVITY OF PRL 26

Substance	Units × 10^6
Control	440
0.001 *M* Cysteine	460
0.001 *M* Glutathione	448
0.003 *M* Ferrous ammonium sulphate	725
0.003 *M* Manganese sulphate	432
0.003 *M* Barium acetate	431
0.006 *M* Calcium chloride	422
0.006 *M* Cobalt sulphate	422
0.003 *M* Magnesium sulphate	421
0.003 *M* Zinc sulphate	397
0.003 *M* Nickel nitrate	326
0.003 *M* Mercuric sulphate	268
0.003 *M* Copper chloride	209
0.003 *M* Silver nitrate	183

The effect of various substances on the activity of a concentrated enzyme preparation of PRL 26 is shown in Table III. The enzyme preparation used for these studies was exhaustively dialyzed to remove any trace metals. The metal was incubated with the enzyme for two hours at room temperature before the activity was measured. The increase in activity on the addition of ferrous ion was quite marked, but the addition of other metals which affect the activity of peptidases, such as cobalt, manganese, and magnesium ions, did not increase the activity of this preparation. The ions of heavy metals such as silver, copper, and mercury inhibited the action of the enzyme, but the effect of reducing agents was negligible.

Earlier observations have shown that peptidases must be activated by metals to obtain maximum hydrolysis. The fact that proteinases also require metals for their activation is noteworthy (4, 14, 15).

Acknowledgment

The author is indebted to Miss J. Corrigal of this laboratory for her technical assistance in this study and to Dr. Wendy M. Dion and Mr. Spencer who supplied the proteolytic enzymes used in this work.

References

1. Adams, E. and Smith, E. L. Proteolytic activity of pituitary extracts. J. Biol. Chem. 191 : 651–664. 1951.
2. Anson, M. L. The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. J. Gen. Physiol. 22 : 79-89. 1938.
3. Berger, J., Johnson, M. J., and Peterson, W. H. The proteolytic enzymes of some common molds. J. Biol. Chem. 117 : 429–438. 1937.
4. Bresler, S. E. and Rozentsveig, N. A. Proteolytic enzymes are metallo-proteins. Biokhimiya, 16 : 84–94. 1951.
5. Crewther, W. G. and Lennox, F. G. Preparations of crystals containing protease from *Aspergillus oryzae*. Nature, 165 : 680. 1950.
6. Dion, W. M. The proteolytic enzymes of microorganisms. I. Survey of fungi and actinomycetes for protease production in submerged culture. Can. J. Research, C, 28 : 577–585. 1950.
7. Ito, Y. On the protease action of *Penicillium notatum*. I. J. Biochem. (Japan), 37 : 51–63. 1950.
8. Johnson, M. J. and Peterson, W. H. The peptidase system of *Aspergillus parasiticus*. J. Biol. Chem. 112 : 25–34. 1935.
9. McConnell, W. B. The proteolytic enzymes of microorganisms. III. Some characteristics of extracellular proteases produced in submerged culture. Can. J. Research, C, 28 : 600–612. 1950.
10. Nelson, N. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153 : 375–380. 1944.
11. Northrop, J. H. The mechanism of the influence of acids and alkalies on the digestion of proteins by papain or trypsin. J. Gen. Physiol. 5 : 263–274. 1922.
12. Northrop, J. H., Kunitz, M., and Herriott, R. M. Crystalline enzymes. 2nd ed. Columbia University Press, New York. 1948.
13. Rivett, R. W., Johnson, M. J., and Peterson, W. H. Laboratory fermentor for aerobic fermentations. Ind. Eng. Chem. 42 : 188–190. 1950.
14. Sagai, K. Protease. VI. Effect of the addition of various salts on tryptic activity. J. Biochem. (Japan); 36 : 91–100. 1944.
15. Snoke, J. E. and Neurath, H. The proteolytic activity of striated rabbit muscle. J. Biol. Chem. 187 : 127–135. 1950.

STUDIES ON THE LEGUME ROOT NODULE BACTERIA

III. GROWTH FACTOR REQUIREMENTS FOR EFFECTIVE, INEFFECTIVE, AND PARASITIC STRAINS[1]

By D. C. Jordan[2]

Abstract

Under the experimental conditions used, amino acids played a very important part in the growth initiation of washed cells of alfalfa – sweet clover rhizobia. There were, however, distinct differences in utilization, both among genetically related mutants, and among other cultures when compared before and after plant passage. None of 15 vitamins, purines, and pyrimidines was able to initiate growth and hence these rhizobia are able to synthesize these compounds when a readily utilizable nitrogen source is present. Because of this fact, the stimulation of these bacteria by yeast extract is probably due, primarily, to the amino acid content. No strain was found able to concentrate free amino acids intracellularly or, when grown in lysine or tyrosine media, to excrete additional amino acids. No differences were found among effective, ineffective, or parasitic rhizobia in biochemical requirements.

Introduction

Many of the investigations pertaining to *Rhizobium* physiology have dealt with growth factors, particularly unidentified ones found in various plant extracts. Some of these factors may be vitamins, and Allen and Allen (1) cite numerous workers who have found certain vitamins to be essential for the growth of a number of rhizobia strains. In comparison, the amino acid nutrition of the nodule bacteria has received only scanty attention and, in many cases, the results are contradictory. The confused state of these amino acid studies may be due to the experimental methods used, including differences in the basal media and the methods of handling the inocula, which in many instances were not washed to remove presynthesized materials. Even more important, however, is probably the disregard for the fact that different strains of the same organism (and even different cells of the same strain) may differ in nutritional requirements.

It is proposed, in the present study, to use only organisms of the alfalfa – sweet clover group and in regard to these particular organisms only a few isolated facts can be found in the literature. Some strains require biotin (20), while others synthesize it (11), whereas thiamine (11) and pantothenic acid (8) are regarded as nonessential. Pohlman (12) reports that *dl*-amino-*n*-butyric acid, *dl*-valine, *d*-glutamic acid, *l*-cystine, *l*-tyrosine, *dl*-phenylalanine, and *p*-aminobenzoic acid can be utilized, but the activity of alanine (11, 17, 19) and glycine (9, 12) is debatable.

Thus far, the biochemical properties of *Rhizobium* have not been linked with nitrogen-fixing efficiency (21). Pohlman (12) suggested that the "tyrosine – melanin" reaction could be used for the detection of effective and ineffective strains, but Almon and Fred (2) disagreed with this proposal.

[1] *Manuscript received April 30, 1952.*

Contribution from the Department of Bacteriology, Ontario Agricultural College, Guelph, Ontario. Part of the program of the Legume Research Committee in Ontario.

[2] *Assistant in research.*

Effective nodule bacteria fix large amounts of molecular nitrogen while ineffective strains fix only minute quantities. Parasitic types fix no detectable nitrogen and in addition may elaborate a phytotoxic substance (6). Such drastic changes in the "normal" physiology of the two latter types conceivably could be detected by the loss (or gain) of the ability to utilize a definite biochemical compound. Obligate parasitism is often defined as a state resulting from the loss of an enzyme system connected with the synthesis of an essential metabolite. This theory could be applied to *Rhizobium* parasitism if it is assumed that the "essential metabolite" is one that is essential for nitrogen fixation rather than for growth. (This supposition becomes even more plausible when it is realized that nitrogen fixation and growth are not necessarily correlated. Fixation is carried on within the nodule even when the rhizobia are in the "resting" or bacteroid stage.) The detection of this important enzyme system and the determination of its absence in parasitic rhizobia would, therefore, aid in the formulation of a rapid biochemical test for such bacteria.

This composite investigation was carried out (*a*) to identify the amino acids, vitamins, and nucleic acid fractions necessary for the growth of washed cells in a synthetic medium, (*b*) to determine whether or not differences in growth factor utilization could be coupled with effectiveness, ineffectiveness, or parasitism, (*c*) to study, by paper partition chromatography, the uptake and excretion of amino acids during growth, and (*d*) to find a synthetic medium that would promote rapid growth of both washed and unwashed inocula.

In this study the term "growth factors" will imply amino acids, vitamins, purines, and pyrimidines.

Methods

All glassware used was thoroughly cleaned by boiling for five hours in 33% nitric acid solution, followed by rinsing 10 times in distilled water and twice in special, double-distilled water, prepared in an all-glass still in the presence of potassium permanganate and silver nitrate. A number of 6–304B photonephelometer cuvettes were cleaned, plugged with cheesecloth-wrapped cotton, and autoclaved. The purified synthetic basal medium used was essentially that of Wilson and Wilson (20) and had the following composition: sucrose, 2.500 gm.; potasium dihydrogen phosphate, 0.500 gm.; ammonium chloride, 0.376 gm.; magnesium sulphate, 0.200 gm.; calcium sulphate, 0.200 gm.; sodium chloride, 0.100 gm.; a trace of ferric chloride; glass-distilled water, 1000 ml. The pH was adjusted to 7.0 before sterilization by Seitz filtration. This latter procedure ensured that the components of the medium were not altered by heat. Ten-milliliter quantities of this base were pipetted into the sterile cuvettes and sterile growth factor solutions added in accurately measured amounts. The final concentrations of the various factors were those designated by Roepke *et al.* (14) and in every instance specially purified water was used in the preparation of the stock solutions.

TABLE I

TABLE SHOWING CODE NUMBERS, SOURCES, AND EFFECTIVENESS OF ALL THE ALFALFA – SWEET CLOVER ORGANISMS TESTED

Strain	Source	Effectiveness
R_{20}	O.A.C. stock culture	Parasitic
R_{20-17}	Colonial mutant of R_{20}	Parasitic
R_{20-27B}	Colonial mutant of R_{20} before plant passage	Effective
R_{20-27A}	R_{20-27B} after plant passage	Effective
R_{20-28B}	Colonial mutant of R_{20} before plant passage	Effective
R_{20-28A}	R_{20-28B} after plant passage	Effective
R_{20-31B}	Colonial mutant of R_{20} before plant passage	Effective
R_{20-31A}	R_{20-31B} after plant passage	Effective
R_{21}	O.A.C. stock culture	Effective
R_{23}	O.A.C. stock culture	Effective
100	Wisconsin stock culture	Effective
105	Wisconsin stock culture	Effective
111	Wisconsin stock culture	Effective
127	Wisconsin stock culture	Ineffective
135	Wisconsin stock culture	Ineffective*

* *According to Dr. O. N. Allen, who so kindly supplied the University of Wisconsin stock cultures, this strain approaches a state of parasitism under conditions of poor host photosynthesis.*

The *Rhizobium* strains used are shown in Table I. The colonial morphology and manner of isolation of the mutants have been previously reported (5). Such mutants, differing from their parent strain in effectiveness, are of particular value in the recognition of biochemical changes connected with parasitism or ineffectiveness, primarily because they are genetically related. Therefore, strain variation is held to a minimum.

Seventy-two-hour slant cultures of these organisms were used to inoculate 10-ml. amounts of a modified mineral salts – yeast extract medium, containing no additional carbon source (22). This lack of carbohydrate served to reduce bacterial gum formation. After incubation for 48 hr. at 25° C. the bacteria were centrifugalized, washed five times in sterile 0.85% saline solution, and suspended in 10 ml. of the same fluid. The saline solution was prepared from the special water and sodium chloride purified by the method of Wilson and Wilson (20). The resulting suspensions were standardized by aseptically adding sterile saline solution so that upon examination in a Coleman spectrophotometer readings of 98% T were recorded at a wave length of 420 mμ.

To the cuvettes of metabolite media 0.2 ml. aliquots of dilute bacterial suspension were added and incubation carried out at 25° C. for eight days. Readings were periodically taken with a Coleman photonephelometer. The turbidity values obtained with this instrument previously had been shown to give excellent agreement with viable bacterial numbers. Inoculated cuvettes containing only basal medium were included in all the series, together with

uninoculated cuvettes which served as "blanks" for the turbidity readings. All readings were recorded in "nephelos" units, using a commercially prepared standard. Tubes showing turbidity at the end of incubation were checked for purity by the Gram stain, the Robinow nuclear stain, as used by Murray *et al.* (10), and by plating out in mannitol – calcium glycerophosphate medium (4). Final pH measurements were taken with a Beckman glass-electrode potentiometer.

The paper partition chromotography method advocated by Woiwod (23) was used to study the uptake of amino acids. R_{21}, R_{20}, R_{20-17}, and R_{20-27B} cultures were grown in Lochhead's yeast extract medium (7) for four-, five-, and six-day periods at 25° C. The cultures were centrifugalized and the supernatants, after reduction to one-quarter of the original volume in dialyzing sacs exposed to a stream of air, were spotted on filter paper sheets and chromatographed. The solvents used were *n*-butanol and acetic acid. Cultures of R_{20}, R_{20-27B}, R_{20-27A}, R_{21}, and R_{23}, after 10 days' incubation in basal medium alone and basal medium plus either lysine or tyrosine, were chromatographed for the detection of amino acids released from the cells by autolysis or by synthesis and subsequent excretion. Rhizobia from the alfalfa, bean, pea, clover, and soybean cross-inoculation groups were grown in 100 ml. amounts of Lochhead's medium and after six days the cells were washed and harvested. These cells were then ground with powdered glass in a motor-driven, ground glass homogenizer and the resulting extracts analyzed by paper chromatography for the presence of free, intracellular amino acids.

Results

The results of the growth factor tests are shown in Table II. Visible turbidity was usually obvious within 24–72 hr. The values observed with any one strain in the various amino acid media are directly comparable one with the other, but are not comparable with the values obtained with other strains because of inevitable variations in inoculum size. All tubes showing turbidity contained only short Gram-negative rods, which exhibited the typical barred condition when examined by the Robinow method. The final pH of these tubes varied between 6.50 and 6.90. The tubes showing no detectable growth were incubated for an additional five-day period and when no change occurred histidine was added. Growth commenced after suitable incubation, showing that the original factors were not bacteriostatic or bactericidal, but were simply not being utilized for growth purposes. None of the vitamins, purines, or pyrimidines used gave any growth, the "nephelos" values being uniformly zero. This group included *p*-aminobenzoic acid, ascorbic acid, biotin, choline, folic acid, inositol, nicotinamide, pantothenate, pyridoxine, riboflavin, thiamine, adenine, guanine, thymine, and uracil.

To eliminate the possibility that growth was due to biotin contamination, washed *Rhizobium* cells were placed in basal medium containing both histidine and avidin. Avidin, a substance found in egg white, combines with biotin and prevents its absorption by cells. Normal growth ensued in every case

TABLE II

TABLE SHOWING FINAL TURBIDITY READINGS, IN "NEPHELOS" UNITS, OBSERVED IN GROWTH FACTOR MEDIA AFTER EIGHT DAYS' INCUBATION

Growth factors	Organisms tested														
	100	105	111	127	135	R_{20}	R_{21}	R_{23}	R_{20-17}	R_{20-27B}	R_{20-27A}	R_{20-28B}	R_{20-26A}	R_{20-31B}	R_{20-31A}
dl-Alanine	18.0	7.5	0.0	0.0	8.5	14.0	13.0	13.0	3.0	40.5	56.5	51.5	64.0	74.5	37.0
l-Arginine	0.0	0.0	0.0	0.0	0.0	13.9	18.0	16.2	0.0	24.2	35.0	58.0	14.5	78.0	19.0
l-Asparagine	27.0	17.0	10.0	2.0	29.5	13.0	15.6	22.2	19.0	24.0	36.0	45.0	72.0	52.0	83.0
l-Aspartic acid	6.0	0.0	0.0	0.0	0.0	20.5	18.6	19.2	3.0*	48.0	54.0	48.0	39.0	48.0	51.0
l-Cysteine	63.0	132.0	149.0	38.0	82.5	10.5	105.0	114.1	32.0	61.0	54.5	51.0	106.0	84.0	92.0
l-Cystine	39.5	83.0	75.5	45.0	86.7	91.5	2.0	1.0	12.0	100.0	59.5	72.0	45.5	105.0	58.0
3,4 *dl*-Dihydroxyphenyl-alanine	66.5	35.0	45.0	33.0	14.0	10.0	16.0	46.5	16.0	17.0	26.5	6.5	18.0	19.0	21.0
l-Glutamic acid	18.5	0.0	0.0	0.0	0.0	21.0	19.8	21.6	3.0*	54.0	51.7	50.5	64.0	52.5	30.0
Glycine	20.0	0.0	25.0	31.6	14.0	65.0	70.0	63.7	20.0	54.4	64.3	84.0	72.0	130.0	92.0
l-Histidine	45.0	22.0	55.0	52.0	83.5	27.6	22.4	24.7	33.0	65.5	75.6	79.0	79.0	87.0	96.0
dl-Leucine	22.0	0.0	0.0	14.0	5.0	8.5	25.4	13.9	6.0	67.5	67.5	86.0	73.0	42.0	101.0
dl-Lysine	21.0	0.0	0.0	0.0	0.0	13.0	19.2	8.0	6.0	36.6	30.4	32.0	41.5	72.5	26.5
dl-Methionine	39.0	5.5	31.0	27.5	53.0	19.8	23.4	18.9	14.0	85.0	67.0	100.0	68.0	82.0	68.0
dl-Phenylalanine	57.0	18.0	11.0	16.0	25.5	42.0	91.4	127.6	194.0	141.5	148.0	41.0	39.0	33.0	10.0
l-Proline	16.0	0.0	0.0	0.0	0.0	24.7	15.3	7.2	0.0	38.6	34.7	27.0	46.0	30.0	62.5
l-Tryptophane	25.5	0.0	8.5	4.6	11.0	21.9	25.8	25.5	22.0	57.0	60.0	59.0	43.0	56.0	67.0
l-Tyrosine	18.0	0.0	0.0	0.0	8.0	0.0	46.0	132.0	35.0	57.0	59.5	124.0	52.0	130.0	130.0
Inoculated base	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Uninoculated base	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Yeast extract	144.0	119.5	150.0	144.0	220.0	184.0	231.0	228.0	206.5	140.0	214.0	215.0	255.0	103.0	266.0

* *Observed after 11 days' incubation.*

and it was concluded that biotin was not essential for the growth initiation of the washed cells. Biotin has also been reported (20) to be stimulatory to growing cells of the nodule bacteria. Consequently washed cells were grown in histidine medium containing 0.0001 μgm. of biotin per milliliter and the growth compared with that observed in histidine medium without biotin. Little, if any, stimulation was observed with the majority of strains as a result of biotin addition.

Paper partition chromatography disclosed the presence of alanine, tyrosine, and the histidine, glycine, glutamic acid, methionine, and leucine groups in the uninoculated yeast extract medium. After six days' growth in this substrate there was no visual difference in the general appearance of the chromatograms, in that one or more of the amino acids were entirely utilized or in that peptides were synthesized and excreted. After only five days' growth, however, it became apparent that R_{20} was utilizing most of the amino acids at a more rapid rate than R_{21}, but this was only a manifestation of the faster growth rate of R_{20}. Tyrosine did not appear to be acted upon to a detectable extent by any strain. No additional ninhydrin–positive materials were released into the growth medium by cells grown in the presence of lysine or tyrosine, nor were free, intracellular amino acids found after cellular disintegration.

Discussion

The results indicate that under the experimental conditions used amino acids play a particularly important role in the growth initiation of washed cells of alfalfa – sweet clover rhizobia. There is a distinct strain difference, however, even with genetically similar mutants, in the utilization of these compounds. Some amino acids are not active with certain strains while others, such as cysteine, cystine, dihydroxyphenylalanine, histidine, methionine, and phenylalanine are consistent in initiating growth. The negative results may, however, be the result of unfavorable pH, temperature, or the presence of sucrose. Distinct changes occurred in the colonial mutants after plant passage, in regard to the growth observed with several amino acids. This parallels the change in the colonial morphology of these mutants after passage through the host plant (5). The utilization of individual amino acids may be interpreted as a method of obtaining ammonia for the synthesis of nitrogenous cell constituents. Evidently washed cells of alfalfa – sweet clover bacteria require an amino nitrogen group for the production of metabolically active ammonia, since no growth occurred when the inorganic nitrogen source (ammonium chloride) in the basal medium was used as the sole source of nitrogen. The fact that such a large number of amino acids were utilized may indicate that in this group of rhizobia these amino acids are linked by transamination mechanisms.

None of the 15 vitamins, purines, and pyrimidines was able to initiate growth and it can be assumed that when a readily utilizable nitrogen source is present these rhizobia synthesize all their essential vitamins and nucleic acid fractions, in addition to nitrogenous cell structural components.

In relation to biotin requirements it has been found (20) that, (*a*) in the absence of biotin most strains grow very poorly and reach a population of about 1/10th the maximum, (*b*) a few strains attain practically maximum growth in its absence, and (*c*) a few strains are unable to grow unless biotin is supplied. Obviously the rhizobia studied in this report belong to the second of the above classifications.

Since growth in 1% yeast extract was extremely good it may be concluded that the stimulation of growth by this material is not a result of vitamin activity. This confirms the results of Werkman (18) who states that yeast extract is active for *R. leguminosarum* not because of vitamins, but because it supplies additional amounts of both nitrogen and energy material. The chromatography results confirm the report of Sadler *et al.* (15) in that the nitrogen of bacto-yeast extract exists in the simpler degradation products of protein decomposition, primarily amino acids. Therefore, yeast extract stimulates these rhizobia mainly as a result of its content of mixed amino acids, although unidentified components also may be important.

The conclusion is reached that a satisfactory synthetic medium for alfalfa – sweet clover bacteria is the medium of Wilson and Wilson supplemented by amino acids. Such a medium, containing histidine, cysteine, and methionine, has been in use in this department for several months and supports adequate growth of washed and unwashed *Rhizobium* cells from at least four cross-inoculation groups. Notable exceptions are the slow-growing strains, which produce only a sparse growth in this particular substrate.

The chromatography experiments agree with the work of Proom and Woiwod (13) and Taylor (16) on Gram-negative bacteria, in that no strain of nodule organism was found to concentrate free amino acids intracellularly.

Unfortunately, no consistent difference was found among effective, ineffective, or parasitic rhizobia, either in the ability to utilize the 32 growth factors or in the behavior of these strains as studied by paper partition chromatography. It must be remembered, however, that, although the role of the host plant has been neglected in this study, it cannot be entirely forgotten, for the state of parasitism may have arisen from a symbiotic conflict which has escaped its natural controls (3). Perhaps it will be only through an intimate study of rhizobia–plant interactions that rapid, biochemical procedures for the detection of ineffective and parasitic strains will be discovered.

Acknowledgments

The author wishes to thank Mr. J. Douglas for his assistance with the chromatographic procedures and Miss Delpha Younie for technical assistance during the preliminary experiments.

References

1. Allen, Ethyl K. and Allen, O. N. Biochemical and symbiotic properties of rhizobia. Bact. Revs. 14 : 273–330. 1950.
2. Almon, L. and Fred, E. B. The production of tyrosinase among various species of rhizobia and related organisms. Zentr. Bakt. Parasitenk, Abt. II, 88 : 302–304. 1933.
3. Caullery, M. Le parasitisme et la symbiose. 2nd ed. Doin et Cie. Paris. 1950.
4. Hofer, A. W. A characterization of *Bacterium radiobacter* (Beijerinck and Van Delden) Lohnis. J. Bact. 41 : 193–224. 1941.
5. Jordan, D. C. Studies on the legume root nodule bacteria. II. The production and behavior of colonial mutants produced by X-ray irradiation. Can. J. Botany, 30 : 125–130. 1952.
6. Jordan, D. C. and Garrard, E. H. Studies on the legume root nodule bacteria. I. Detection of effective and ineffective strains. Can. J. Botany, 29 : 360–372. 1951.
7. Lochhead, A. G. Departmental communication. Unpublished data.
8. McBurney, C. H., Ballen, W. B., and Williams, R. J. Pantothenic acid and the nodule bacteria legume symbiosis. Proc. Natl. Acad. Sci. U.S. 21 : 301–304. 1935.
9. Müller, A. and Stapp, C. Beiträge zur Biologie der Leguminosenknöllchenbakterien mit besonderer Berücksichtigung ihrer Artverschiedenheit. Arb. biol. Reichsanstalt Land-u. Forstw. Berlin-Dahlem, 14 : 455–554. 1925.
10. Murray, R. G. E., Gillen, D. H., and Heagy, F. C. Cytological changes in *E. coli* produced by infection with phage T_2. J. Bact. 59 : 603–615. 1950.
11. Nielsen, N. and Johansen, G. Untersuchungen über biologische Stickstoffbindung. V. Untersuchungen über die Wirkung verschiedner Wuchsstoffe auf 4 Stämme von *B. radicicola*. Compt. rend. trav. lab. Carlsberg. Sér. physiol. 23 : 173–193. 1941.
12. Pohlman, G. G. Changes produced in nitrogenous compounds by *Rhizobium meliloti* and *Rhizobium japonicum*. Soil Sci. 31 : 385–406. 1931.
13. Proom, H. and Woiwod, A. J. The examination, by partition paper chromatography, of the nitrogen metabolism of bacteria. J. Gen. Microbiol. 3 : 319–327. 1949.
14. Roepke, R. R., Libby, R. L., and Small, Margaret, H. Mutation or variation in *Escherichia coli* with respect to growth requirements. J. Bact. 48 : 401–412. 1944.
15. Sadler, W., Eagles, B. A., and Pendray, G. Cheese-ripening studies. II. The nitrogen requirements of the lactic acid bacteria. Biochem. J. 26 : 1532–1535. 1932.
16. Taylor, Shirley. The assimilation of amino-acids by bacteria. 3. Concentration of free amino-acids in the internal environment of various bacteria and yeasts. J. Gen. Microbiol. 1 : 86–90. 1947.
17. Walker, R. H., Anderson, D. A., and Brown, P. E. Physiological studies on *Rhizobium*. II. The effect of nitrogen source on oxygen consumption by *Rh. meliloti*, *Rh.. trifolii*, and *Rh. phaseoli*. Soil Sci. 38 : 207–217. 1934.
18. Werkman, C. H. Vitamin effects in the physiology of microorganisms. J. Bact. 14 : 335–347. 1927.
19. West, P. M. and Wilson, P. W. Biotin as a growth stimulant for the root nodule bacteria. Enzymologia, 8 : 152–162. 1940.
20. Wilson, J. B. and Wilson, P. W. Biotin as a growth factor for rhizobia. J. Bact. 43 : 329–341. 1942.
21. Wilson, P. W. Symbiotic nitrogen-fixation by the Leguminoseae. Botan. Rev. 3 : 365–399. 1937.
22. Wilson, P. W. Respiratory enzyme systems in symbiotic nitrogen fixation. I. The "resting cell" technique as a method for the study of bacterial metabolism. J. Bact. 35 : 601–623. 1938.
23. Woiwod, A. J. A technique for examining large numbers of bacterial culture filtrates by partition chromatography. J. Gen. Microbiol. 3 : 312–318. 1949.

APHANOMYCES CLADOGAMUS DRECH., A CAUSE OF DAMPING-OFF IN PEPPERS AND CERTAIN OTHER VEGETABLES[1]

By C. D. McKeen[2]

Abstract

A species of *Aphanomyces* was isolated from damped-off pepper seedlings which had been grown in a sandy loam compost soil under glass. This fungus was obtained from up to 8% of the damped-off pepper seedlings. The morphological characteristics of this fungus corresponded so closely with the descriptions of *Aphanomyces cladogamus* Drech., which Drechsler isolated from diseased tomato rootlets, that the two fungi are considered to be the same. Reduction in stands of pepper seedlings resulted from both pre-emergence and post-emergence damping-off, when seeds were planted in soil inoculated with isolates of the fungus. Characteristic symptoms of postemergence attacks consisted of black lesions on the hypocotyl frequently extending into the bases of the cotyledons, and death of affected seedlings usually occurred. *A. cladogamus* was found to cause a considerable amount of damping-off in tomato, eggplant, radish, and spinach as well as in pepper. It attacked Spanish onions only slightly, but lettuce, pea, and muskmelon not at all. Except in spinach, *A. cladogamus* showed little tendency to colonize root tissues, whereas hypocotyl and cotyledonary tissues were highly vulnerable.

Introduction

During the course of investigations of the various phases of damping-off in peppers, numerous isolations from diseased seedlings were made. A water mold belonging to the genus of *Aphanomyces* was occasionally obtained. With few exceptions the diseased tissue that yielded the water mold yielded no other fungus, thus indicating it to be the primary pathogen. The appearance of diseased seedlings indicated a type of pathogenicity not much different from that displayed by several species of *Pythium* and *Rhizoctonia solani* Kuehn. Primarily because of the interest aroused by the occurrence of this fungus as a damping-off pathogen, its identification and a study of its parasitic capabilities were undertaken. A preliminary account of these findings was presented earlier (6). In the present paper, the various phases of the investigation are presented in greater detail.

Literature Review

In 1926, Drechsler (1) isolated from diseased rootlets of tomato seedlings in Virginia, U.S., a water mold tentatively identified as *Aphanomyces euteiches* Drech. In 1929, the same author in a more extensive publication (2), pointed out that the fungus obtained from tomatoes in 1926 showed rather definite morphological dissimilarities to the pea parasite *A. euteiches*. Thus, the fungus obtained from tomato was considered to be an undescribed species and, accordingly, was named *Aphanomyces cladogamus* Drech. Drechsler (3) reported that from the diseased roots of spinach plants collected in New Jersey, in May of 1930, and also from the discolored roots of stunted spinach plants

[1] *Manuscript received May 8, 1952.*

Contribution No. 1176, Division of Botany and Plant Pathology, Science Service, Department of Agriculture, Ottawa, Canada.

[2] *Associate Plant Pathologist, Laboratory of Plant Pathology, Harrow, Ontario.*

collected in Virginia, in November of 1932, he isolated a water mold considered to be the same as he isolated earlier from tomato. In 1931, he also obtained numerous cultures of the same fungus from the discolored roots of stunted flax plants in Wisconsin. In 1945, Mix (8) reported a rootrot of lettuce, pepper, and eggplant seedlings caused by a species of *Aphanomyces* which he did not identify. The trouble occurred in two localities in New Jersey where these crops were being grown under glass. Whereas the damage to lettuce was light, heavy damage to peppers was reported. Mix found that, when lettuce was planted in soil inoculated with the *Aphanomyces* sp. isolated from pepper seedlings, many seeds failed to germinate, and further that a number of seedlings developed rootrot later, with oospores present in the browned cortex of the roots. A brief summary of Mix's findings was reported in 1947 by Miller and Wood (9).

Experimental Details and Results

In the text that follows, the fungus in question will be referred to as *Aphanomyces cladogamus* Drech. Evidence for this conclusion will be presented later.

Disease Symptoms in Pepper Seedlings

Both the pre-emergence and the postemergence damping-off phases of this trouble have been responsible for reducing stands of pepper seedlings. In seedbed soils heavily infested with *A. cladogamus*, the former has been observed to reduce stands by as much as 40%. The postemergence phase of the disease is, however, the most apparent one and has been observed to reduce stands by as much as 50% (Fig. 16).

The infected tissue of emerging pepper seedlings is soft and tan-colored. At this stage the symptoms are often indistinguishable from those caused by either *Pythium ultimum* Trow or *Rhizoctonia solani* Kuehn, except that the invaded tissue, as it dries, assumes a dark brown to coal black color. Later symptoms, i.e., after the hypocotyl has elongated sufficiently to free the cotyledons from the soil, are, however, strikingly different from those caused by the two commonly occurring, above-mentioned, damping-off pathogens. A blackening and necrosis of the hypocotyl develop and spread into the bases of the cotyledons. Frequently before the black lesion becomes evident externally the invaded portion of the cortical cylinder of the hypocotyl shows as a dark streak when viewed in transmitted light. Although a lesion may extend the entire length of the hypocotyl, it is frequently more restricted to the upper regions of the hypocotyl, especially in slightly older seedlings. The hypocotyl often collapses near the point of attachment of the cotyledons (Fig. 1). Often a lesion extends along one side of the hypocotyl causing it to curve, occasionally through 180°. Seedlings so affected often show partial or complete necrosis of the cotyledon on the lesioned side of the hypocotyl.

In blackroot of sugar beets (4, 7) caused by *Aphanomyces cochlioides* Drech., a chronic phase of the disease is often found long after the acute damping-off phase is past. In peppers, only the acute damping-off phase has been found. After the seedlings have formed the first pair of true leaves, no further attack

of this host has been observed. Moreover, stunted pepper seedlings that have survived an earlier attack have been observed to develop into sturdy plants.

Isolation of the Fungus

When tissue of damped-off pepper seedlings plated on potato dextrose agar (P.D.A.) and on corn meal agar failed to yield the causal fungus, the method of isolation of *Aphanomyces* sp. and members of related genera described by Drechsler (2) and McKeen (7) was used and proved satisfactory. A brief description of the technique employed is as follows: the infected seedlings are thoroughly washed in tap water and with the aid of a camel's hair brush all soil particles are removed. Small pieces of infected tissue are then placed in Petri plates containing sterilized water. Usually within 24 hr. a profuse growth of fungus mycelium is observed to grow out into the water. After adequate mycelial growth is secured, the pieces of infected tissue are removed, and are blotted thoroughly between sheets of filter paper to remove free water. Transfers are then made to plates of nonacidified corn-meal agar that were poured three or four days previously. A slab of harder agar is placed on top of the infected material and is pressed firmly with a scalpel blade to expel any pockets of trapped air. When strands of phycomycetous hyphae appear on the top surface of the agar slab, transfers of a few hyphal tips are made to other plates of agar, the same covering technique being used. Frequently two or three such transfers are found necessary to free cultures from bacterial contamination. The practice of using agar plates poured three or four days prior to isolation aids in the exclusion of bacteria from their intimate association with the fungal hyphae.

Stock cultures of *A. cladogamus* were carried on slants of corn meal agar or in tubes on steam sterilized pepper seedling tissue.

Occurrence of A. cladogamus in Compost Soils

Since no member of the genus of *Aphanomyces* has been reported previously as a pathogen of peppers in Ontario, it seemed necessary to find out the extent to which it was involved as a damping-off pathogen. To provide this information, sufficient compost soil to fill three flats was obtained from each of two commercial greenhouses where damping-off of peppers was extensive in 1950. One thousand pepper seeds of the Pennwonder variety were planted in each flat. The experiment was run at normal greenhouse temperatures in April of 1950. The flats of soil were sprinkled with water once or twice daily to keep the soil moderately wet on the surface. Five hundred damped-off seedlings from each of the two soils were brought into the laboratory to determine the causal organism. After the writer became familiar with the characteristics of this fungus, it was found only necessary to use the first part of the isolation procedure to make this determination. Accordingly, identifications were made after seedlings were allowed to remain in Petri plates containing sterilized water for 24 to 48 hr.

A. cladogamus was obtained from 5 and 8%, respectively, of the damped-off pepper seedlings from the two compost soils.

Cultural and Morphological Characteristics of the Fungus

(a) Growth and Viability in Culture

A. cladogamus was found to grow well in culture both on P.D.A. and corn-meal agar, producing a thin weft of appressed, colorless to faintly whitish mycelium. In macroscopic appearance, cultures of the fungus on the same medium showed a high degree of uniformity. Sexual organs were produced on corn-meal agar, but not on P.D.A. Whereas the fungus remained viable on P.D.A. for only a few weeks, cultures were kept alive on corn-meal agar for six or seven months between transfers. Stock cultures of the fungus were also carried safely for seven months in tubes containing sterilized pepper seedlings. Only rarely were stock cultures carried on any substrate viable after eight months regardless of the temperature of storage.

(b) Mycelial Characteristics

The hyphae are long and sparingly branched. Branches arise virtually at right angles. In addition to the ordinary branches, some hyphal elements produce short diverticulate spurs or hooks. This latter type of branch develops predominantly on the extramatrical mycelium when infected tissue is placed in water. Furthermore, this type of branching is formed just prior to the development of, and in proximity to, the sexual organs.

(c) Asexual Reproduction

Asexual reproduction can be readily induced either by placing an infected seedling in water or by covering with water, the mycelium growing on agar. Usually in less than 10 or 12 hr. the protoplasmic contents of some of the hyphae that grow out into the water become broken up into short segments (Figs. 2 and 3). These sinuous hyphae show little or no tendency to taper toward the apex and occasionally range up to 3 mm. in length. Ultimately the tips of these hyphae dissolve and the separate protoplasmic units flow rapidly out of these hyphae, which now serve as evacuation tubes, into the water, and instantly assume a spherical shape to become nonmotile zoospores. A characteristic feature of the fungus is the frequent occurrence on the more distal portion of the efferent hyphae of several short lateral branches (Figs. 3 and 4), often three or four and more rarely as many as six or seven in number. These short branches often serve as alternate channels of egress and usually function only after zoospores have escaped for a considerable time from the terminal opening. The number of zoospores escaping from a single orifice varies from 10 to several hundred. On the bottom of a Petri plate beneath an infected seedling that has been in a water culture for 12 or more hours, a cloudy white area comprised of thousands of encysted zoospores is frequently observed.

The zoospores vary from 6 to 17.5 μ (average 8 to 9μ) in diameter. A group of encysted zoospores is shown in Fig. 5. Zoospores show only a slight tendency to cling together after they are discharged. Although the motile

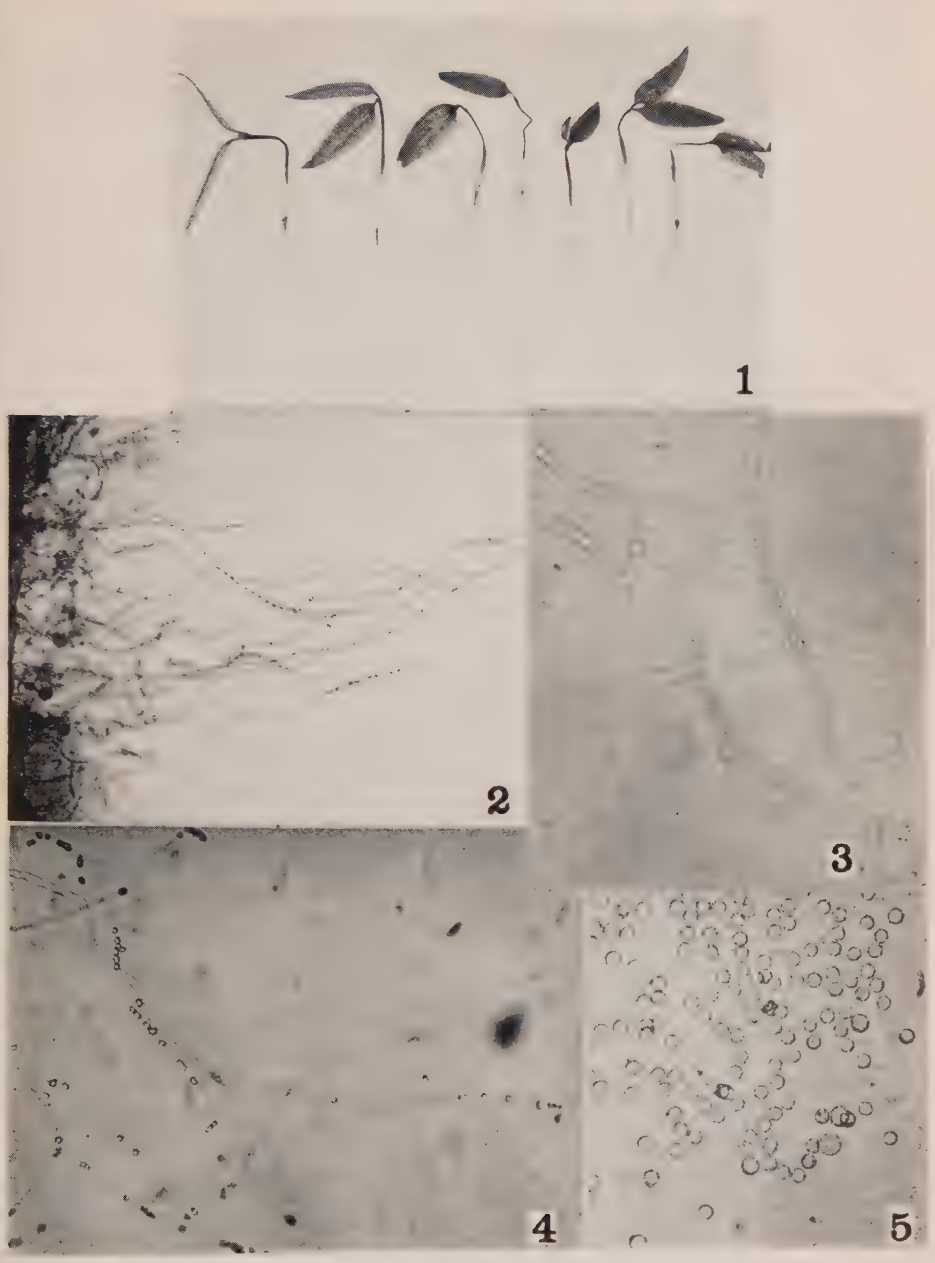


FIG. 1. Pepper seedlings showing black lesions on hypocotyl caused by *Aphanomyces cladogamus* Drech. FIG. 2. Extramatrical mycelium extending out into the water from a diseased seedling. Some of the hyphae are functioning as zoosporangia. ×64. FIG. 3. Efferent hyphae containing protoplasmic units about to be discharged as zoospores. ×350. FIG. 4. Efferent hyphae, one of which shows short lateral branches functioning as alternate channels of egress for zoospores. ×120. FIG. 5. A group of encysted zoospores. ×270.

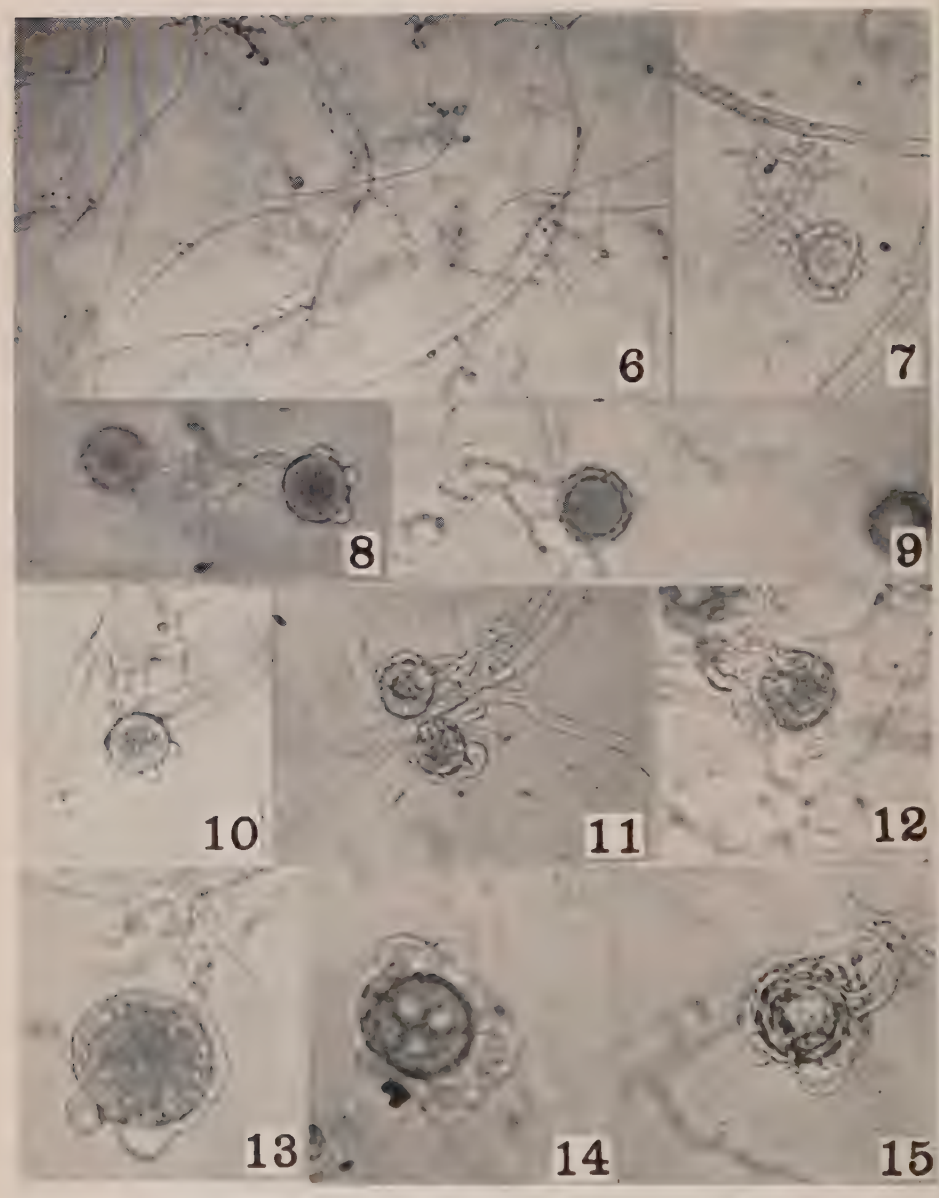


FIG. 6. Extramatrical mycelium showing much branched structures that give rise to sexual organs. ×80. FIG. 7. A branched structure bearing a partially developed oogonium. ×340. FIG. 8. Two oogonia developing in close proximity on a branched structure. ×340. FIG. 9. Antheridium showing diclinous origin. ×340. FIG. 10. Oogonium showing three antheridia attached. ×340. FIG. 11. Two oogonia showing partial involvement by antheridia. ×340. FIG. 12. The inflated part of an antheridium applied to an oogonium bearing a fingerlike projection. ×340. FIG. 13. An antheridium showing the insertion of a septum at some distance below the inflated part. ×340 and enlarged. FIG. 14. An oogonium showing five antheridia attached. ×340 and enlarged. FIG. 15. A fertilized oogonium. Note the relatively short stalk. ×340.

stage occurs in this fungus, as in other members of this genus, it must be mentioned that, in the writer's experience, rather few motile spores appeared in comparison with the vast quantity of encysted zoospores present.

(d) Sexual Reproduction

When a diseased seedling is placed in water the sexual organs make their appearance usually after the first crop of zoospores has been released from the efferent hyphae. However, on seedlings showing advanced stages of decay, sexual organs are often produced without the intervention of the asexual stage. Sexual organs are produced in abundance within the decaying host tissues, but, since their development was more readily observed on the extramatrical mycelium in water cultures, their morphological characteristics were studied mostly on the latter medium. The commencement of the sexual stage is indicated by the development of much-branched structures that arise at intervals on the extramatrical mycelium (Fig. 6). At one or occasionally two points on each branched structure, a spherical oogonium develops (Figs. 7 and 8). As the oogonium enlarges, antheridial elements may become differentiated from parts of the same branched structure (monoclinous origin) and come into intimate association with the oogonium (Figs. 8, 10, and 11). Antheridia may also arise (androgynously) from nearby branches that have their origin from the same axial hypha that bears the oogonial stalk or they may arise (diclinously) from an adjacent hypha (Fig. 9). The writer experienced no difficulty in finding antheridia having the three different types of origin, although the last type was the least common. There is considerable involvement of the oogonium by the antheridia (Figs. 10, 11, 12, 13, and 14). A characteristic feature of this fungus is the frequent insertion of a septum delimiting the anteridium at some distance below the inflated part (Fig. 13). On the inflated portion of some of the anteridia, a fingerlike projection (Fig. 12) from 3 to 4μ in diameter and ranging up to 13μ in length was found. Other antheridia bear a distal prolongation of the inflated part, ranging up to 14μ long. There are usually two or three, occasionally four and more rarely five antheridia (Fig. 14) attached to each oogonium. The average dimensions of the inflated part of the antheridium were found to be 13μ in length by 10μ in diameter.

The oogonia are terminal on short or longer branches, subspherical and smooth, ranging from 21 to 31.5μ (average 25.8 μ) in diameter (Fig. 15), and are provided with a wall of irregular inner contour. The average diameter of the oospores was found to be 19.1μ, and each oospore contained an eccentrically placed reserve globule. The germination of a few oospores was observed when the surfaces of old cultures were covered with water. They produced a germ tube which later functioned as a zoosporangium.

Variability Among Isolates of Aphanomyces

During the course of these investigations four isolates of the fungus designated as *A*, *B*, *C*, and *D*, were studied critically with respect to morphology and pathogenicity. The following differences were noted. Isolate A was

consistently slower by several hours than the other three isolates in producing zoospores, when methods of inducing the asexual stage were carried out. Isolate B consistently had three or four antheridia attached to each oogonium, whereas the other three isolates consistently had two or three. Another variable character was the degree of pathogenicity expressed. Isolate D was found to cause only 50% as much damping-off in pepper seedlings as the other three isolates. These slight morphological and pathogenic differences displayed by the four isolates were construed as representing variations within a species that is comprised of variously differentiated forms.

Identity of the Fungus

The morphology of the fungus isolated from peppers and described in the foregoing sections corresponded so closely with the descriptions of the fungus that Drechsler (2) isolated from diseased tomato rootlets that the two fungi would appear to be identical. Slight variability in the number of antheridia attached to each oogonium as well as slight differences in the average dimensions of the oogonia and oospores of the two fungi are not regarded as being significant. Thus, the fungus here described is identified as *Aphanomyces cladogamus* Drech.

Pathogenicity

(a) Comparative Pathogenicity of A. cladogamus and A. cochlioides

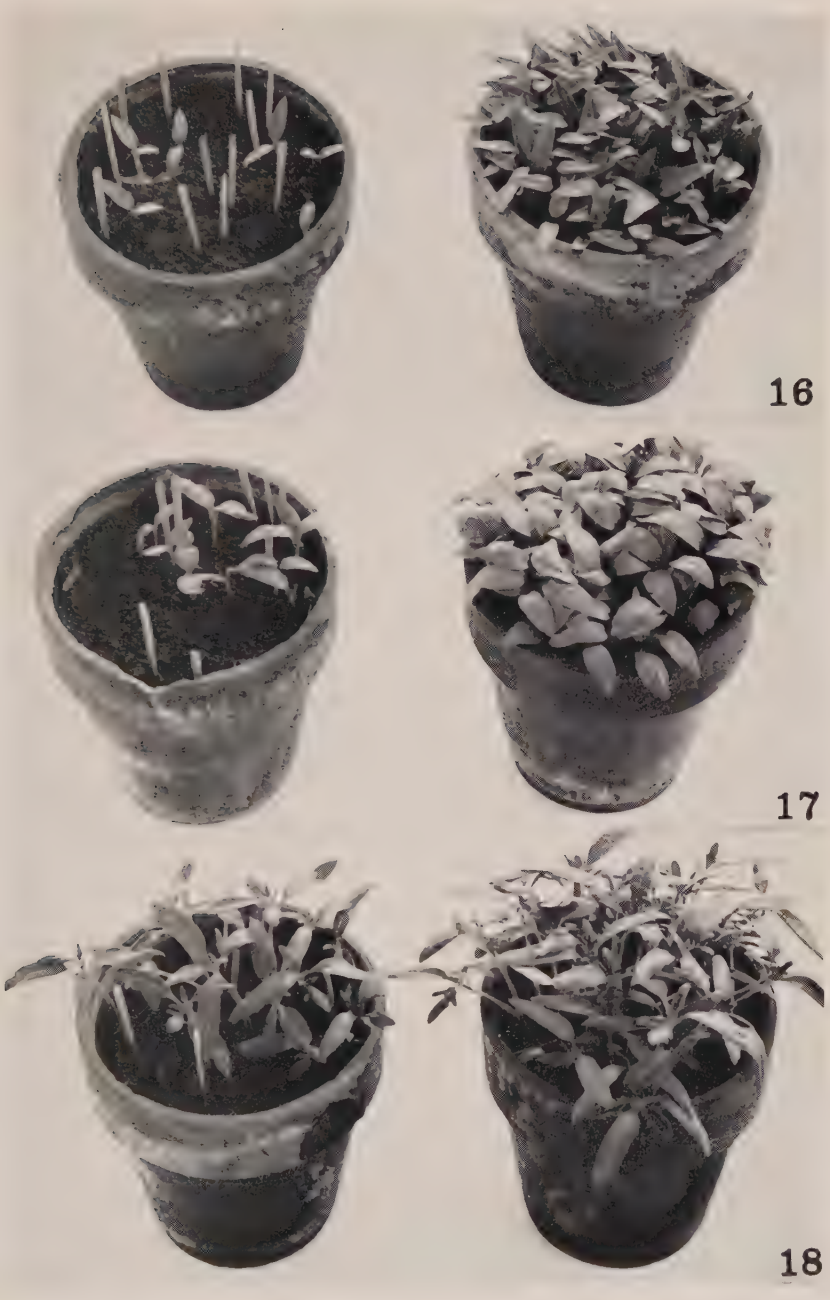
Since a culture of the sugar beet water mold (*A. cochlioides*) was available at the laboratory, it seemed worthwhile to compare its pathogenicity with that of *A. cladogamus* on both peppers and sugar beets. For this experiment, eight-day-old corn-meal agar cultures of each fungus were diced and were added to the surface of steam sterilized soil in 4-in. pots. The inoculum was then covered with a thin layer of steamed soil.

Fifty seeds of either pepper or sugar beets were planted in each of the inoculated pots of soil and were covered to the appropriate depth with more steamed soil. As controls, seeds of both hosts were planted in pots of non-inoculated steamed soil. Results of this test are shown in Table I.

TABLE I

COMPARATIVE PATHOGENICITY OF *A. cladogamus* AND *A. cochlioides* TO PEPPER AND SUGAR BEET SEEDLINGS

Host	Percentage stand in steamed soils		
	Inoculated with *A. cladogamus*	Inoculated with *A. cochlioides*	Noninoculated (check)
Sugar beet	58	0	98
Pepper	50	77	94



FIGS. 16, 17, and 18. Pepper, eggplant, and tomato seedlings, respectively, planted in pots of steamed soil; left, inoculated with *Aphanomyces cladogamus*, right, check (non-inoculated soil). Toothpicks represent the incidence of postemergence damping-off.

As Table I shows, *A. cochlioides* was much more pathogenic to sugar beets than was *A. cladogamus*. Conversely, the latter was appreciably more pathogenic to peppers than the former. *A. cladogamus* reduced the stand in peppers only slightly more than that in sugar beets.

(b) Comparative Pathogenicity of Aphanomyces cladogamus, Rhizoctonia solani, and Pythium ultimum

From comparative parallel pathogenicity tests with *R. solani*, *P. ultimum*, and *A. cladogamus*, it was found that *A. cladogamus* was incapable of causing as much damping-off in peppers as the other two fungi.

(c) Host Range of A. cladogamus

Inasmuch as the few species of *Aphanomyces* that have been isolated and identified to date have been fairly restricted in their host range, it seemed desirable to ascertain the pathogenicity of *A. cladogamus* to several vegetables. Accordingly, two tests were conducted in a manner similar to that described in the preceding section. The following vegetables were used: radish, pea, eggplant, tomato, lettuce, Spanish onion, and muskmelon.

A. cladogamus was found capable of causing considerable pre-emergence and postemergence damping-off in tomato, radish, spinach, and eggplant, as well as in pepper (Figs. 16, 17, and 18). In an earlier report by the writer (6), Spanish onion was listed among the vegetables not susceptible to attack. More recent work, however, has shown the fungus to be capable of causing a slight amount of damping-off in this host. The radish plant showed much more pre-emergence damping-off than postemergence damping-off. In tomato, as in pepper, the postemergence phase of the attack lasted from 5 to 10 days, depending upon the growth of the host as influenced by environmental conditions. In all hosts the parts attacked were primarily the cotyledons and the hypocotyl. In spinach there was more discoloration and colonization of primary root tissue than in the other vegetables. Lettuce, pea, and muskmelon were not attacked.

Host–Parasite Relations

Kendrick (5) reported that *Aphanomyces raphani* entered the radish seedling through a natural wound made where a secondary root emerged from the primary root. McKeen (7), working on black root of sugar beets, found that *Aphanomyces cochlioides* readily penetrated the hypocotyl of the sugar beet seedling, and he postulated that the fungus may have entered through the stomata of the hypocotyl. The writer found numerous incipient lesions at the base of the hypocotyl of both pepper and tomato seedlings with no evidence of *A. cladogamus* in the roots of the infected plants. Further, a high percentage of the pepper seedlings was attacked before secondary roots made their emergence. This observational evidence would indicate that *A. cladogamus* can readily enter the seedlings through the hypocotyl and that it does not rely upon entrance through natural root wounds.

Drechsler (3) isolated *A. cladogamus* from discolored roots of stunted spinach plants. Mix (8) reported an *Aphanomyces* sp. as causing a rootrot of peppers, eggplant, and lettuce. The writer, however, found no evidence of *A. cladogamus* being an aggressive root parasite. Infected pepper seedlings in which mycelium of the fungus had colonized extensively both the hypocotyl and cotyledons often showed an apparently healthy root system. Moreover, when pepper seedlings showing much diseased aboveground parts were placed in water, the fungus was observed to grow prolifically out into the water from the hypocotyl and cotyledonary tissues, whereas only traces of fungus mycelium were found associated with root tissue. Oospores were produced in abundance in the former tissues but were relatively few in number in the latter.

Discussion

The results from the foregoing experiments showed that *A. cladogamus* was limited in its parasitism, as are many other damping-off pathogens, to the attack of emerging and young seedlings. Moreover, only primary tissues were susceptible to attack. The fungus showed little tendency to be actively parasitic upon root tissues, except at the upper region of the primary root of spinach, where some browning and necrosis was observed. The report by Mix (8) of a serious root rot of lettuce, pepper, and eggplant seedlings in New Jersey, U.S.A., caused by *Aphanomyces* sp., would indicate that a different species of this genus was involved.

The fact that *A. cladogamus* was isolated from damped-off pepper seedlings from different greenhouse compost soils would indicate that it may be a normal component of the flora of many soils. Also, the fact that Drechsler isolated this fungus once from diseased tomato rootlets, and on two other occasions from diseased roots of stunted spinach and flax plants from widely separated localities in U.S.A., would support this view. It seems likely, however, that it may never occur in large numbers in many soils for two chief reasons. First, the fact that *A. cladogamus* can attack only young seedling tissues of certain vegetables, many of which are started in greenhouses or plant beds, would afford it little or no opportunity to build up in field soils. Second, in southwestern Ontario, as in many other vegetable-growing areas, the practice of using compost soil for greenhouse seedbed purposes only once would provide no opportunity for its increase through long association with a susceptible crop. Thus, there would seem to be little likelihood of *A. cladogamus* becoming a predominant component of seedbed soils and hence this fungus will probably never become a serious damping-off pathogen. Recent tests of the comparative pathogenicity of *A. cladogamus*, *P. ultimum*, and *R. solani* have shown the first fungus to be the least pathogenic of the three.

Acknowledgment

The writer wishes to express his appreciation to Dr. L. W. Koch for helpful criticism of the manuscript.

References

1. DRECHSLER, C. Two water molds causing tomato rootlet injury. J. Agr. Research, 34 : 287-288. 1927.
2. DRECHSLER, C. The beet water mold and several related root parasites. J. Agr. Research, 38 : 309-361. 1929.
3. DRECHSLER, C. Occurrence of a species of *Aphanomyces* on roots of spinach and flax. Phytopathology, 25 : 14-15. 1935.
4. HILDEBRAND, A. A., McKEEN, W. E., and KOCH, L. W. Row treatment of soil with tetramethylthiuram disulphide for control of blackroot of sugar-beet seedlings. I. Greenhouse tests. Can. J. Research, C, 27 : 23-43. 1949.
5. KENDRICK, J. B. The black root disease of radish. Indiana Agr. Expt. Sta. Bull. 311 : 1-32. 1927.
6. McKEEN, C. D. A species of *Aphanomyces* causing damping-off in peppers and in certain other vegetables. Abstract. Phytopathology, 24 : 16. 1952.
7. McKEEN, W. E. A study of sugar beet rootrot in southern Ontario. Can. J. Research, C, 27 : 284-311. 1949.
8. MIX, A. J. *Aphanomyces* rootrot of lettuce, pepper and eggplant seedlings in Northern New Jersey. Plant Disease Reptr. 29 : 649–650. 1945.
9. MILLER, P. R. and WOOD, J. I. An evaluation of certain phases of the emergency plant disease prevention project. Plant Disease Reptr. Suppl. 167 : 6 and 18. 1947.

STUDIES IN FOREST PATHOLOGY

IX. *FOMES IGNIARIUS* DECAY OF POPLAR[1]

By C. G. Riley[2]

Abstract

Decay of *Populus tremuloides* Michx. and *P. grandidentata* Michx. caused by *Fomes igniarius* var. *populinus* (Neuman) Campbell was studied at the Petawawa Forest Experiment Station, Ont., in stands up to 70 years of age. In eight $\frac{1}{10}$th acre sample plots representing stands 60 to 70 years old, 28.6 to 69.2% of the trees were infected; the percentage of decay in the gross merchantable cubic volume was 3.2 to 14.3 in the decayed trees, and 0.9 to 8.1 in decayed and sound trees considered together. Equally variable results were obtained in younger age classes. No relation between decay and site could be established on the basis of actual cubic volume, but when the trees were measured in board feet a slightly higher percentage of cull occurred on the poorer sites, owing at least partly to the smaller trees. Net periodic increment continued to increase in the 60–70-year age class on favorable sites. Sporophore-bearing trees died in the second and third years after being girdled, and the original number of mature living sporophores was reduced to 13% six years after girdling. On similar trees which were felled and left intact on the ground during the same period the number of sporophores increased. Artificial inoculations in both sapwood and heartwood of living trees resulted in the development of typical decay from which the pathogen was re-isolated. Observations on 5 to 18 sporophores from May 4 to June 30 of the following year proved that sporulation of *F. igniarius* is continuous from early spring to late autumn. There is not necessarily any period of inactivity during which new tube layers are formed, though irregular periods of one to several days without sporulation are common. Sporulation is favored by high relative humidity and high temperature. The lowest temperature at which sporulation was observed was 40° F.

Introduction

The names "poplar" and "aspen" as used in this report, refer without distinction to trembling aspen (*Populus tremuloides* Michx.) and large-tooth aspen (*P. grandidentata* Michx.). These two species occur in essentially even-aged, frequently pure stands, and also in mixture with other species. No distinction is made between them locally for purposes of silviculture or utilization, and no difference could be discovered with respect to the relationships investigated.

Until recent years poplar has received little attention from a commercial point of view, having been considered as a weed tree with negligible utilization value. However, as a result of the greatly increased use of this wood in the manufacture of match splints, pulp, and other products, the native aspens are now considered among the most important forest species within economically accessible range of manufacturing plants. In many such localities the supply is rapidly approaching depletion.

One of the most difficult problems to be met in the production of poplar arises from the common occurrence of heartwood decay caused by *Fomes igniarius* var. *populinus* (Neuman) Campbell (21) (referred to throughout

[1] *Manuscript received February 19, 1951, and, as revised, July 17, 1952.*

Contribution No. 58 from the Division of Forest Biology, Science Service, Department of Agriculture, Ottawa, Canada. This paper constitutes part of a thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy in the University of Toronto, May, 1946.

[2] *Officer-in-charge, Laboratory of Forest Pathology, Saskatoon, Sask.*

this report simply as *F. igniarius*). The age at which management plans should provide for crop removal depends largely upon the condition of the stands with respect to this defect.

The primary object of this investigation was to determine the relation of decay to site and age. The relative effectiveness of two methods of eradicating sporophores, and sporulation of the fungus were also studied.

The investigation was conducted at the Petawawa Forest Experiment Station, Ont., approximately 95 miles northwest of Ottawa. This area, which lies in the Algonquin–Laurentides Section of the Great Lakes–St. Lawrence forest region (13), is part of a glaciated plateau having a general altitude above sea level of about 700 ft., with a few prominent ridges rising above that level. The underlying rock is Laurentian gneiss and granite, and the glacial till covering most of the hillsides is very sandy (1). The principal forest sites and forest types of the region have been described in detail by Heimburger (14) and Sisam (26). The forest stands are generally even-aged, having become established following fires. The principal cover types include stands of red pine and white pine; poplar; jack pine; white birch and white pine; spruce and balsam fir; and a few spruce swamps and stands of tolerant hardwoods (26).

Decay in Relation to Site and Age of Stand

Methods

Decay was studied in relation to site and age of stand on $\frac{1}{10}$th acre sample plots representing all poplar sites and age classes that occur at the Petawawa Forest Experiment Station. All poplar trees on the plots were analyzed except in some of the younger stands where smaller samples were taken. The characteristic appearance of some of the stands is illustrated in Figs. 1 to 5.

Tree ages were counted on the stumps which were 6 to 12 in. high, depending on the degree of butt swell. As poplar stands ordinarily originate as root suckers following fire or cutting operations, early growth is rapid, and age counted on low stumps generally corresponds with total age.

The boles were bucked into 4-ft. lengths, and inside-bark diameter and decay diameter were measured at each cut. Decay measurements included all marginal discolorations associated with the decay (Fig. 6). The gross cubic volumes of the 4-ft. bolts were computed by means of Smalian's formula (10), which was used also to compute the volume of decay where this occurred as a core throughout the length of the bolt. Where decay occurred at only one end of a bolt, its volume was computed as for a cone.

The relation of decay to sporophores was studied and a separate paper on this subject has been published (23).

Decay in Relation to Site

The site index used in this study is the average annual height increment of the dominant and codominant trees on each plot. The basic data for the plots are summarized in Table I.

TABLE I

BASIC PLOT DATA

Plot number	Number of trees analyzed	Average age of dominant and codominant trees, years	Average height of dominant and codominant trees, ft.	Site index
1	21	64.0	89.9	1.40
2	28	68.4	71.2	1.04
3	34	70.1	78.7	1.12
4	22	60.4	82.2	1.36
5	26	70.5	77.4	1.10
6	21	43.3	89.9	2.08
7	27	70.1	92.7	1.32
8	28	67.3	89.0	1.32
10	15	66.9	97.3	1.45
11	13	66.6	91.4	1.37
12	72	22.0	44.8	2.04
13	23	23.0	60.4	2.63
14	43	22.0	54.8	2.49
15	34	34.0	73.9	2.17
16	96	21.0	40.3	1.92
17	49	36.5	76.0	2.08
18	43	37.3	69.8	1.87
19	79	44.3	79.4	1.79
20	96	36.1	61.0	1.69
21	60	45.8	69.7	1.52

In order to minimize the influence of factors other than those of site, trees in which decay had originated in large wounds such as lightning scars, fire scars, and mechanically-caused wounds were rejected. *F. igniarius* is essentially a wound parasite which gains entrance into living trees only where openings occur through the protective layer of living bark. These openings are of two kinds: wounds such as those mentioned above, and dead branches and branch snags resulting from normal self-pruning. The latter are inherent in the tree, and their number and size depend partly upon such factors as stand density and rate of growth which are closely related to site quality. Their presence may be considered as a normal hazard with respect to infection by heart rot fungi. On the other hand, wounds caused by agents such as fire, lightning, and man usually are not closely related to site, and trees bearing large wounds of this nature are abnormally predisposed to the attacks of heart rot fungi.

A second class of trees that were rejected was comprised of those in which the volume deviated widely above or below the appropriate multiple of the standard error, according to the method of Wright (30). However, only two trees were rejected on this basis.

In the following analyses the amount of decay is expressed in terms of percentage of infected trees, percentage of merchantable cubic volume[1] in infected trees only, and percentage of merchantable cubic volume in all trees.

[1] *Merchantable volume: the gross volume of wood between stump and top at 3 in. diameter.*

FIG. 1. View near Plot 10 in 67-year-old stand. These trees were exposed to full-height view by a recent logging operation. Average height of dominant and codominant trees, 97.3 ft. FIG. 2. View on Plot 15 in 34-year-old stand. FIG. 3. Characteristic view in 67-year-old stand shown in Fig. 1. *F. igniarius* sporophores on trees in foreground.

FIG. 4. View on Plot 14, in 22-year-old stand. FIG. 5. Plot 7 in 70-year-old stand. Trees bucked into 4-ft. lengths. FIG. 6. A continuous series of 2-ft. sections from a poplar tree severely decayed by *F. igniarius*. These sections comprise the entire bole, 36 ft. long and 8 in. in diameter inside bark at top. Age of tree 67 years. Diameter at breast height 11.7 in. Total height 78 ft.

In Table II the plot data are grouped in 10-year age classes, within each of which they are arranged in order of site index. By this means any appreciable degree of correlation that may exist between decay and site should be indicated by an increasing or decreasing trend in the values of decay within each age class. No such trend is apparent in the table. On the contrary, the pronounced irregularity of the decay values indicates that, if any relationship does exist, it must be an extremely broad one that is not likely to be discernible except on comparison of large samples drawn from stands representing extremes of site quality.

TABLE II

DECAY DATA ARRANGED IN ORDER OF SITE INDEX WITHIN 10-YEAR AGE CLASSES

Age class	Plot number	Site index	Percentage of infected trees	Percentage of decay	
				Infected trees only	All trees
21–30	16	1.92	Nil	—	—
	12	2.04	Nil	—	—
	14	2.49	Nil	—	—
	13	2.63	Nil	—	—
31–40	20	1.69	1.7	4.8	0.1
	18	1.87	13.1	4.3	0.7
	17	2.08	7.1	1.4	0.1
	15	2.17	5.9	1.1	0.1
41–50	21	1.52	13.6	5.3	0.7
	19	1.79	38.9	4.8	2.3
	6	2.08	14.3	7.3	1.0
51–60	4	1.36	26.3	7.3	1.3
61–70	2	1.04	37.0	14.3	4.3
	5	1.10	28.6	3.2	0.9
	3	1.12	41.2	8.3	4.1
	8	1.32	45.8	10.9	5.2
	7	1.32	62.5	7.8	5.2
	11	1.37	69.2	10.4	8.1
	1	1.40	29.4	6.5	2.9
	10	1.45	64.3	4.8	3.0

It was considered possible that the local variations in amount of decay might be related to the presence or absence of abundant sources of inoculum in close association with young developing stands. The four plots, Nos. 12, 13, 14, and 16, which were located in an extensive 22 to 23-year-old stand at considerable distances from any older, sporophore-bearing trees, were entirely free of decay. The occasional occurrence of sporophore-bearing trees in the vicinity of the plots indicated that the stand had attained a susceptible age. A special sample plot was analyzed near the edge of the stand in close proximity to a large group of overmature trees bearing many large *F. igniarius* sporophores. Here, as in the other plots in this stand, no decay was found. It thus appears that, in this stand at least, the proximity of sporophores was not a factor in determining the amount of decay present.

Decay in Relation to Age of Stand

In the absence of evidence that the amount of decay is appreciably influenced by local variations of site, this possibility was disregarded in further treatment of the data. The decay data were rearranged (Table III) in order of average age of the plots to examine the correlation between decay and age of stand.

TABLE III

DECAY DATA ARRANGED IN ORDER OF AGE

Plot number	Average age	Percentage of infected trees	Percentage of decay	
			Infected trees only	All trees
16	22	0	—	—
12	22	0	—	—
14	22	0	—	—
13	23	0	—	—
15	34	5.9	1.1	0.1
20	36	1.7	4.8	0.1
17	36	7.1	1.4	0.1
18	38	13.1	4.3	0.7
6	43	14.3	7.3	1.0
19	44	38.9	4.8	2.3
21	44	13.6	5.3	0.7
4	59	26.3	7.3	1.3
1	64	29.4	6.5	2.9
8	67	45.8	10.9	5.2
10	67	64.3	4.8	3.0
11	67	69.2	10.4	8.1
2	60	37.0	14.3	4.3
3	70	41.2	8.3	4.1
7	70	62.5	7.8	5.2
5	70	28.6	3.2	0.9

TABLE IV

REGRESSION EQUATIONS, WITH STANDARD ERROR OF ESTIMATE AND COEFFICIENT OF CORRELATION FOR PERCENTAGE OF INFECTED TREES, AND PERCENTAGE OF DECAY, OVER AGE OF STAND

	Percentage of infected trees	Percentage of decay	
		In infected trees only	In all trees
Regression equation[1]	$Y = -29.37+1.106X$	$Y = -1.59+0.15X$	$Y = -3.63+0.11X$
Standard error of estimate	14.2	2.78	1.66
Coefficient of correlation	0.75	0.60	0.71

[1] Y = *per cent;* X = *age of stand.*

Regression equations were computed (12) for percentage of infected trees, percentage of decay in infected trees only, and percentage of decay in all trees, over age of stand. These equations are shown in Table IV and the corresponding regression lines are shown in Figs. 7, 8, and 9. By substituting age of stand for X in the appropriate regression equation, the actual or anticipated percentage of decay may be calculated for any stand at any age between 30 and 70 years. Table V shows values calculated in this manner at 10-year intervals. Percentages calculated by this method may be applied to present or predicted gross merchantable volume inventories in order to estimate net volumes.

TABLE V

SMOOTHED AVERAGES OF PERCENTAGE OF INFECTED TREES AND PERCENTAGE OF DECAY AT 10-YEAR INTERVALS IN AGE OF STAND. CALCULATED BY MEANS OF REGRESSION EQUATIONS SHOWN IN TABLE IV

Age	Percentage of infected trees	Percentage of decay	
		Infected trees only	All trees
30	3.81	2.91	Nil
40	14.87	4.41	0.77
50	25.93	5.91	1.87
60	36.99	7.41	2.97
70	48.05	8.91	4.07

The mean gross merchantable volume, decay volume, and net merchantable volume per tree were calculated with respect to the better sites only, since it is only with respect to the most suitable sites for the species concerned that such information is likely to have practical value in forest management. Five plots (Nos. 20, 21, 2, 5, and 3, see Table II) were eliminated for this purpose. In addition, the four plots in the 21 to 30-year age class were excluded since the merchantable volume in these was negligible and decay was practically absent.

A freehand curve (Fig. 10) of mean gross merchantable cubic volume per tree over age was prepared by Ezekiel's method (12). The standard error of estimate of this curve is 6.46 and the index of correlation is 0.81. Table VI

TABLE VI

MEAN GROSS MERCHANTABLE VOLUME, DECAY VOLUME, AND NET MERCHANTABLE VOLUME, IN CUBIC FEET PER TREE, IN RELATION TO AGE

Age	Gross merchantable volume	Decay volume		Net merchantable volume	
		Infected trees only	All trees	Infected trees only	All trees
40	9.0	0.4	0.1	8.6	8.9
50	13.7	0.8	0.3	12.9	13.4
60	20.0	1.5	0.6	18.5	19.4
70	29.1	2.6	1.2	26.5	27.9

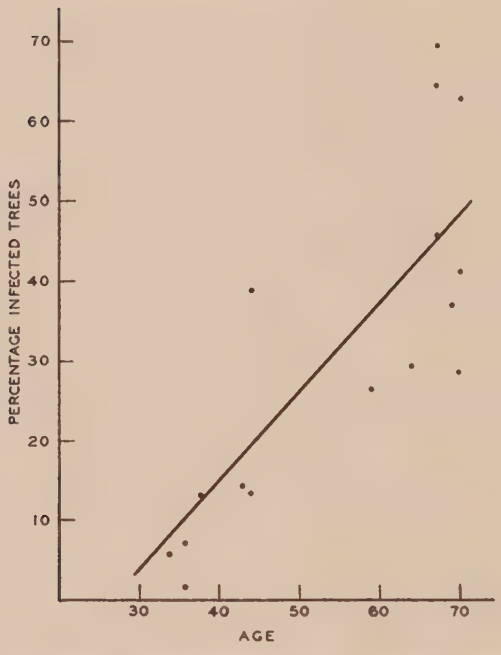


FIG. 7. Percentage infected trees in relation to age. Plotted points represent sample plots.

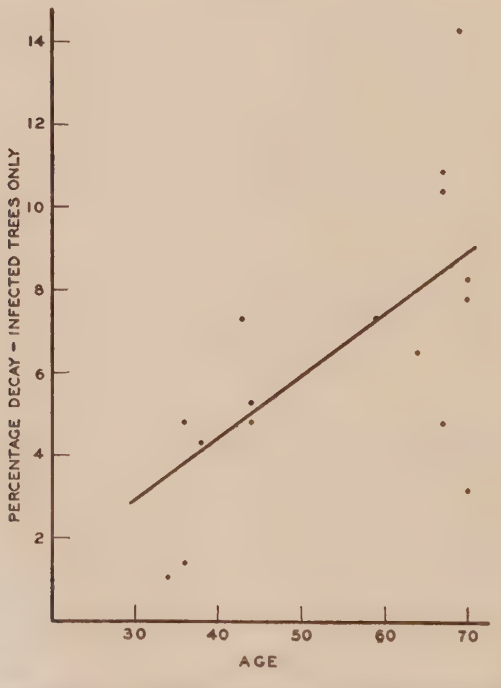


FIG. 8. Percentage decay, infected trees only, in relation to age. Plotted points represent sample plots.

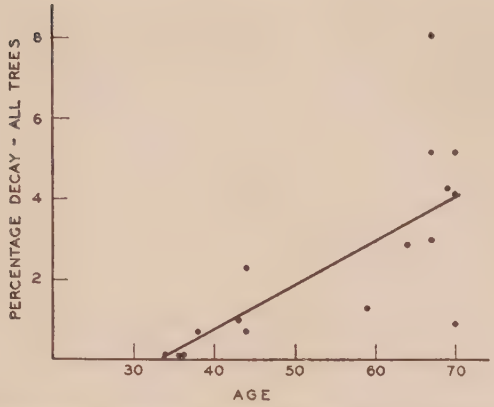


FIG. 9. Percentage decay including all trees (sound and infected) in relation to age. Plotted points represent sample plots.

shows gross merchantable volume as indicated by this curve at 10-year intervals. This table also shows corresponding values for decay (derived from Table V) and net merchantable volume. Curves representing these results are included in Fig. 10.

Fig. 10 and Table VI show that net merchantable volume continues to increase with increasing age in stands up to 70 years old, even if infected trees only are considered. However, economic considerations require that the *rate* of increase be taken into account when determining rotation or age of harvesting. Accordingly, the periodic increment in 10-year periods as derived from the data in Table VI is shown in Table VII and Fig. 11. The results indicate no decrease in the rate of net increment up to 70 years of age.

In order to add to the practical value of this investigation the decay–age relationships were worked out in terms of board feet (Doyle log rule, as authorized in Ontario) in addition to the results already presented in terms of cubic volume. Only the plots within the 60-to-70-year age class contained a sufficient number of trees of sawlog size to warrant analysis in terms of board

TABLE VII

PERIODIC INCREMENT (IN CUBIC FEET) OF GROSS MERCHANTABLE VOLUME, DECAY VOLUME, AND NET MERCHANTABLE VOLUME. AVERAGE PER TREE

10-Year periods	Periodic increment of gross merchantable volume	Periodic increment of decay volume		Periodic increment of net merchantable volume	
		Infected trees only	All trees	Infected trees only	All trees
40–50	4.7	0.4	0.2	4.3	4.5
50–60	6.3	0.7	0.3	5.6	6.0
60–70	9.1	1.1	0.6	8.0	8.5

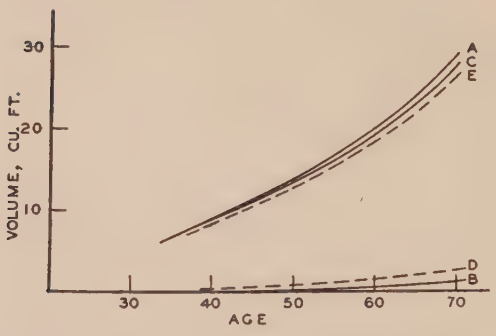


FIG. 10. Volume–age relationships. *A*. Mean gross merchantable volume, all trees. *B*. Mean decay volume, all trees. *C*. Mean net merchantable volume, all trees. *D*. Mean decay volume, infected trees only. *E*. Mean net merchantable volume, infected trees only.

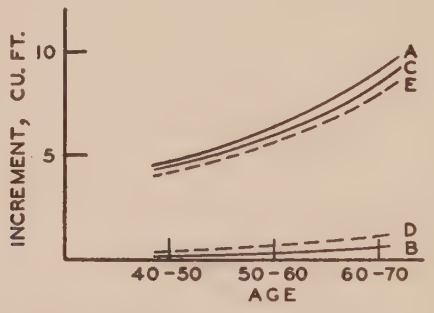


FIG. 11. Periodic increment. *A*. Gross merchantable volume, all trees. *B*. Decay, all trees. *C*. Net merchantable volume, all trees. *D*. Decay, infected trees only. *E*. Net merchantable volume, infected trees only.

feet. Table VIII indicates the average gross, cull, and net board-foot volumes per tree, and the percentage deducted because of decay in this age class. The table shows the average for all sites included in the study, and also for the "better sites" considered separately, to which reference has already been made.

Thus far in this report, only decay caused by *Fomes igniarius* has been discussed. While this fungus is responsible for most of the heartwood decay of poplar, it is by no means the only one attacking the wood of the living trees. *Polyporus dryophilus* var. *vulpinus* (Fries) Overh. was responsible for decay in many trees on Plot 21 (poor site) and fewer on Plot 19 (medium site) in a 44-year-old stand. This fungus is a wound parasite that aggressively attacks trees of low vigor. The associated wounds were caused by sunscald and frost cracks. The decay passes through a white pocket stage and develops into a light-brown stringy rot which, upon shrinking, tends to leave a hollow stem. The advanced decay usually extends throughout the greater length of the trunk and also encroaches into the sapwood.

Pholiota adiposa Fries was responsible for occasional small amounts of butt rot. *Armillaria mellea* (Vahl ex Fries) Quél. was observed attacking poplar trees within the general locality, though it was not found on any of the sample plots.

TABLE VIII

AVERAGE VOLUME PER TREE (BOARD FEET, DOYLE RULE) AND PERCENTAGE DEDUCTED FOR DECAY IN THE 60- TO 70-YEAR AGE CLASS. (AVERAGE AGE 66.9 YEARS)

	On all sites	On "better sites" only
All trees:		
Basic number of trees	141	105
Av. gross vol. (bd. ft.)	51.01	60.71
Av. net vol. (bd. ft.)	42.66	50.95
Av. vol. deducted for decay (bd. ft.)	8.35	9.76
Av. vol. deducted for decay (percentage of gross vol.)	16.4	16.1
Decayed trees only:		
Basic number of trees	61	47
Av. vol. deducted for decay (percentage of gross vol.)	31.4	29.6

Discoloration of otherwise apparently sound wood is very common in poplar. A dull reddish stain typically appears in the vicinity of knots caused by dead branches. Stain is invariably associated with wounds. Heartwood is frequently stained with various shades of dull red, brown, or olive green, which may be intermixed. These discolored regions sometimes extend for considerable distances above or below typical *F. igniarius* decay (Fig. 6) which has no definite line of delimitation at its longitudinal extremities. Numerous cultures on agar from stained wood indicated the presence of various fungi and bacteria. *F. igniarius* was not indicated at distances of more than two or three inches from visibly decayed wood longitudinally, or approximately one-quarter of an inch laterally. No serious attempt was made to identify the other organisms, but two fungi were identified as belonging to the genera *Torula* and *Corticium*. Black (3) lists 10 species of fungi which were identified in association with root and butt rots of poplar in northern Ontario, and six species associated with trunk rots.

Persistence of Sporophores on Girdled and Felled Trees

An experiment was conducted for the purpose of determining the period during which poplar trees may continue to support living sporophores of *Fomes igniarius* following girdling as compared with felling. The location was in the general vicinity of Plots 9 and 10, described earlier in this paper. This was a good site on low flat ground approaching swampy conditions in wet seasons. The sound poplar had been removed the previous year, resulting in a dense ground cover of ferns, shrubs, and young trees. The trees used in the experiment were dominant and codominant trees in a stand in which the dominants averaged 66 years of age, 91.4 ft. in height, and 12.8 in. in diameter at breast height. They bore from 1 to 25 sporophores each.

Methods

Eleven living trees were girdled by chopping away bark and wood to a depth of approximately 1 to 1½ in. Nine additional trees were felled and left otherwise intact. The sporophores were classified into two categories designated as "mature" and "immature". The former comprised those that had developed a pore surface, and the latter those that had not. Sporophores high up on standing trees were examined by means of field glasses. The experiment was begun in late September, and annual re-examination were made at approximately the same date for six years.

Results

One year after girdling the crowns of all trees were still normal in appearance. At the end of the second year one tree was dead and the other 10 had "thin" crowns with fewer, smaller, and paler leaves than the ungirdled, neighboring trees. All were dead at the end of the third year. After six years most of the smaller branches had broken off, parts of the bark had become loosened and were falling away, and some of the trees had broken off at the point of girdling.

New sporophores continued to appear on both girdled and felled trees throughout the six-year period of observation, and these tended to compensate numerically for the death of original sporophores. Further, the number of

TABLE IX

SURVIVAL OF SPOROPHORES (*Fomes igniarius*) AND PRODUCTION OF NEW ONES ON GIRDLED AND ON FELLED POPLAR TREES, IN RELATION TO PERIOD SINCE TREATMENT

Period since treatment, years	Original mature sporophores		Original, plus new sporophores			
			Mature		Immature	
	Number	Per cent	Number	Per cent	Number	Per cent
On girdled trees						
0*	105	100	105	100	18	100
1	95	90	97	92	18	100
2	88	84	88	84	15	83
3	87	83	87	83	14	78
4	79	75	79	75	22	122
5	50	48	51	49	26	144
6	11	10	14	13	1	6
On felled trees						
0*	91	100	91	100	25	100
1	80	88	80	88	53	212
2	75	82	75	82	45	180
3	73	81	91	100	29	116
4	71	78	80	88	82	328
5	67	74	87	96	76	304
6	64	70	92	101	59	236

* *Initial observation.*

mature sporophores was augmented continually by the growth of the immature ones. Table IX indicates the number of original sporophores, the degree of survival, and the fluctuation of numbers, in relation to the period since girdling or felling.

The data indicate a decidedly higher rate of mortality and fewer new sporophores on girdled than on felled trees. Of the original mature sporophores on girdled trees 10% were alive at the end of six years, and the growth of the immature sporophores increased this figure to 13%. On the felled trees 70% of the original mature sporophores survived, while new growth increased the net total to 101% and, in the case of immature sporophores, to 236% of the original number.

The principle of removing diseased trees as a measure of control requires consideration of the possibility of infection by spores carried by wind from sources outside of the stand. This possibility is partly dependent upon the length of time during which spores may remain viable. The viability of spores was tested after various periods of storage during which they were exposed to the air in the laboratory on dry microscope slides. Freshly caught spores germinated freely when incubated at room temperature on standard potato-dextrose or malt agar, or on agar containing poplar sawdust decoction. Practically no germination occurred on nonnutrient agar or when the spores were immersed in, or floating on, tap water or distilled water. On nutrient agar approximately 90% of the spores germinated after three days of storage and 50% after six days; occasional spores germinated after 13 days of storage.

Inoculation Experiments

Published descriptions of *F. igniarius* decay commonly refer to advanced stages of the disease and fail to mention the characteristics of very young infections. Experiments involving inoculation of poplar trees with *F. igniarius* were conducted for the purpose of studying the disease in its early stages.

Methods

The trees selected for these experiments were apparently healthy, dominant and codominant *P. tremuloides* in well-stocked stands.

Inoculum was prepared and used in the following manner. Pure cultures were obtained by inoculating standard malt agar slants with context tissue of *F. igniarius* sporophores from living poplar. Cylindrical plugs made of poplar wood, approximately $\frac{3}{4}$ in. long and $\frac{5}{16}$ in. in diameter, were autoclaved and placed on the surface of the cultures in the test tubes. After the fungus had become established in the plugs, these were used for inoculation by inserting a single plug into a hole bored in the tree with a $\frac{3}{8}$ in. bit. The holes were closed with stoppers which were made as required, from living, healthy, maple wood. These were carefully fitted and trimmed flush with the bark.

In order to determine the results of inoculation, it was necessary to fell the tree, remove a length containing the inoculated part, and split it open for examination (Figs. 12, 13). The presence or absence of the pathogen was determined by the standard method of culturing on malt agar.

Results

Following is a summary of inoculations made in May, 1940, and the results as determined in September, 1941, after two growing seasons. Eight inoculations and four controls were made within the sapwood of 45-year-old trees. Typical decay developed at six of the inoculations (Fig. 12) and *F. igniarius* was re-isolated from each. No decay was associated with the other two inoculations or the controls, and these failed to yield the pathogen in culture. Three inoculations and three controls were made in the heartwood of 10-year-old trees. Typical decay developed (Fig. 13) and *F. igniarius* was re-isolated from each of the inoculations, while the three controls yielded negative results.

In the sapwood infections the pocket of typical *F. igniarius* decay was distinguishable on the radially-split sections for distances of 0.8 to 2.4 in. above and below the point of infection. In the radial and tangential directions the decay extended only slightly beyond the length and the width of the inoculum plug. The decay that resulted from the inoculation of heartwood extended approximately eight inches above and below the point of infection and involved nearly the entire heartwood region in cross section.

In the sapwood infections the typical decay was quite punky or "cheesy", though firm, and of practically the same color as normal sapwood from which it was distinguishable more by its peculiarities of fracture than by its color. On being split radially, the decayed wood tends to break "across the grain" leaving surface irregularities that parallel and expose the medullary rays (Fig. 12). This never occurs in sound wood, on the split surface of which all irregularities run parallel to the wood fibers and vessels. In some instances the decayed region was partly delimited by zone lines, such as are typically associated with *F. igniarius* decay. These were most prominent on the radial and tangential sides of the decay pocket.

In the heartwood infections the symptoms were as described above, but the decay was more advanced (Fig. 13). On a sawn cross section the surface of decayed wood is rough and dull as compared with the smooth bright surface of healthy wood. On the end of a log advanced decay may be partly delimited by thin dark zone lines, outside of which there is commonly a faint grayish band one-quarter of an inch or more wide (Fig. 6). Decayed wood usually becomes slightly darker after it is exposed.

Associated with all inoculation wounds including the controls was a region or zone of reddish brown stain in the sapwood. Similar stain is generally associated with wounds of all kinds in poplar. Numerous organisms were obtained as a result of cultural tests, but none was constantly associated with the stain.

Sporulation

Studies were conducted on the seasonal duration of sporulation and the influence of meteorological factors on the rate of sporulation in *F. igniarius* under natural conditions on living poplar in the forest.

FIG. 12. A successful inoculation with *F. igniarius* in sapwood of a 45-year-old tree. FIG. 13. Successful inoculation with *F. igniarius* in heartwood of 10-year-old tree. Note advanced decay bounded by typical dark lines. The wooden plug that contained the inoculum may be seen in the base of the hole.

Methods

The basic data were obtained by means of spore deposits on glass slides which were fixed beneath sporophores as close to the pore surface as possible without actually touching it. The slides were collected and replaced with clean ones at regular intervals of time. The density of the spore deposit at the end of each interval was used as an index of the rate of sporulation.

Precautions were taken to avoid disturbing the sporophores under observation or interfering with their normal environment. This requirement rendered it impracticable to control certain potential sources of error, probably the most important of which was the carrying away of spores by wind before they reached the surface of the slide. In view of the fact that heavy spore deposits were obtained during periods of continuous wind, it appeared that this source of error was not unduly serious. The loss of spores by wind could have been overcome by constructing wind-proof shelters around the sporophores and spore traps, as was done by Borisov (4). This, however, would constitute a decided departure from natural conditions, probably affecting humidity and temperature in addition to air currents, and thus defeating the purpose of the investigation. Once the spores contact the surface of the slide they adhere so firmly that a dry spore deposit can withstand a considerable amount of rubbing without being seriously disturbed. Buller (7) made a similar observation with respect to spores of the Hymenomycetes generally.

The method of determining the sporulation index of a spore deposit was to average the number of spores counted in 10 sample fields selected at random from the densest part of the spore deposit. This method proved very satisfactory for sporulation indices up to 500 per sample field. In heavier deposits than this the spores were more than one layer deep and could not be counted by this method.

Daily Fluctuation of Sporulation

Preliminary observations indicated that sporulation is favored by warm, humid weather. In order to study this relationship observations were made on nine sporophores at 3-hr. intervals during the 54-hr. period between 11.00 a.m., August 5, and 5.00 p.m., August 7.

The results showed that the rate of sporulation followed the same general pattern of fluctuation in all of the nine sporophores. There were two peaks and two depressions of sporulation daily—the peaks in the morning and evening, and the depressions during the day and during the night. This is illustrated by the graph in Fig. 14, which represents the average of the sporulation indices at each observation. In order to examine this behavior in relation to relative humidity and temperature, graphs of these factors during the same period were superimposed upon the graph of sporulation. It will be noted that the trends of relative humidity and temperature were always opposed, the former rising to a peak at night and declining to a depression during the day, while in the latter the reverse was true. The interrelationships of the three graphs are interpreted as follows. During the day sporulation was favored by high temperature, but was retarded by low relative humidity;

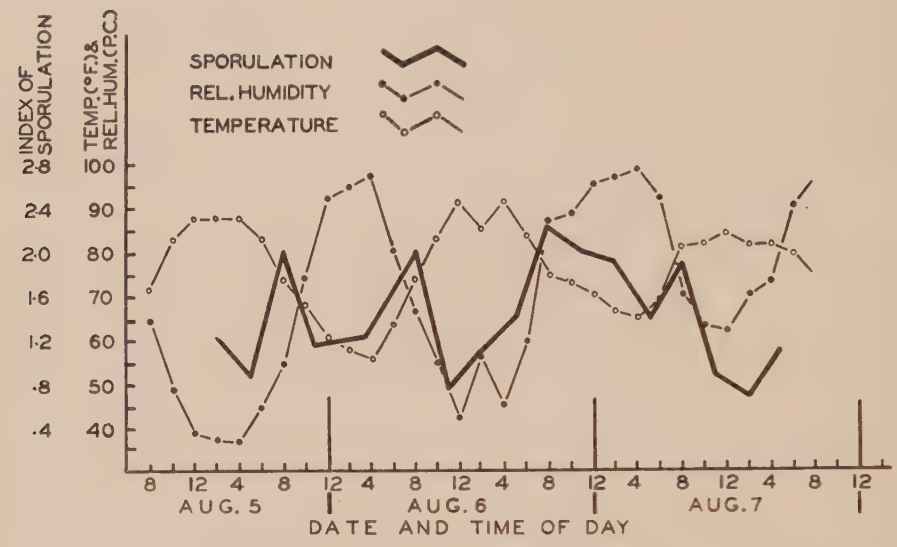


FIG. 14. Sporulation recorded at 3-hr. intervals, in relation to temperature and relative humidity.

during the night while relative humidity was favorable, sporulation was retarded by low temperature. The peaks in the graph of sporulation each morning and evening mark the occurrence of the most favorable combination in the opposing trends of the two factors.

Seasonal Fluctuation of Sporulation

The seasonal fluctuation of sporulation was studied in relation to relative humidity and temperature by means of continuous observations for more than a year on a group of 5 to 18 sporophores. The host trees were all *Populus grandidentata*, 9 to 11 in. in diameter, in a mixed stand, on an average site.

Observations were begun on May 4, 1940, using five sporophores. The number of sporophores was increased to 18 on June 13. With the exception of two periods of six and three days in May, all spore trap slides were changed daily at 8.00 a.m., till September 4, a period of four months. Thereafter spore traps were placed under five sporophores for 24-hr. periods whenever opportunities occurred during warm weather. The latest observation was on November 22, after which continuous winter weather prevailed. Beginning on March 27 of the following year, spore traps were again placed under the same five sporophores in each period of warm weather till the end of April, after which daily observations were resumed till the end of June.

The daily indices of sporulation for each of the 18 sporophores during July and August are graphically represented in Fig. 15. These graphs illustrate the following characteristics. Although spores were liberated on every day on which observations were made from May 4 to Nov. 13, no single sporophore was continuously active throughout the season. During July and August the most constant producer (Graph No. 3) was totally idle for only a single day.

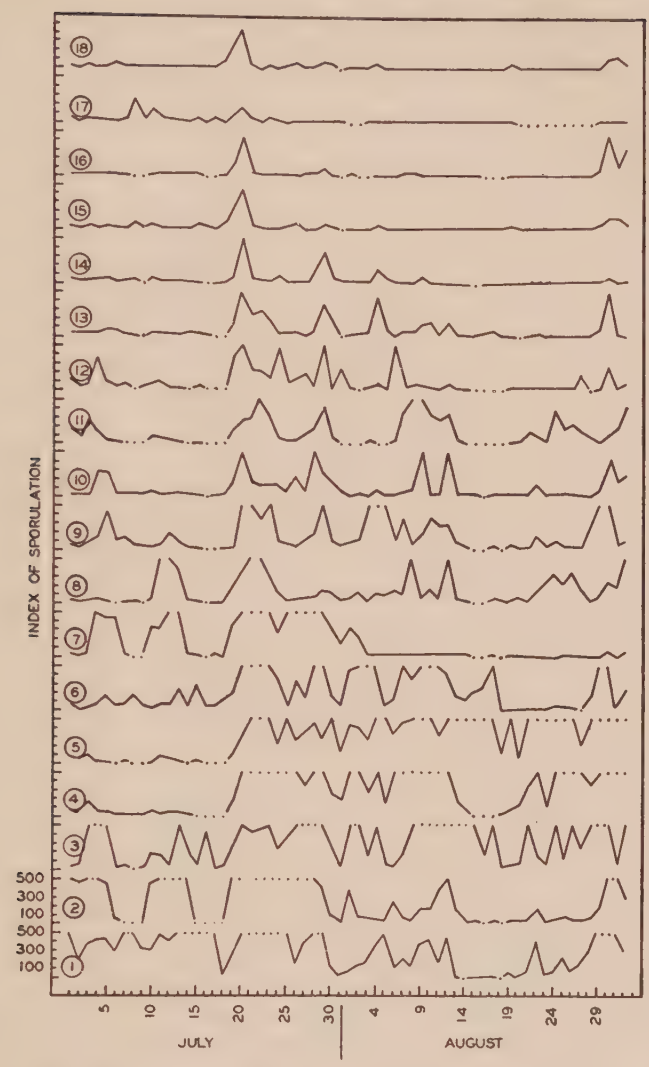


FIG. 15. Daily index of sporulation of 18 sporophores during July and August, 1940. The dots at the lower extreme of each graph (zero) indicate no sporulation on the corresponding dates. Dots at the upper extreme (500) indicate sporulation index of 500 or higher.

Other sporophores had as many as six periods of inactivity, the two longest of which were of nine and five days' duration. There was extreme variation among the sporophores with respect to spore-producing capacity in a given period or throughout the season. Thus, certain graphs (arranged toward the bottom of Fig. 15) indicate heavy sporulation most of the time throughout the two-month period, while others (toward the top of the figure) indicate generally feeble activity. A given sporophore may change abruptly from con-

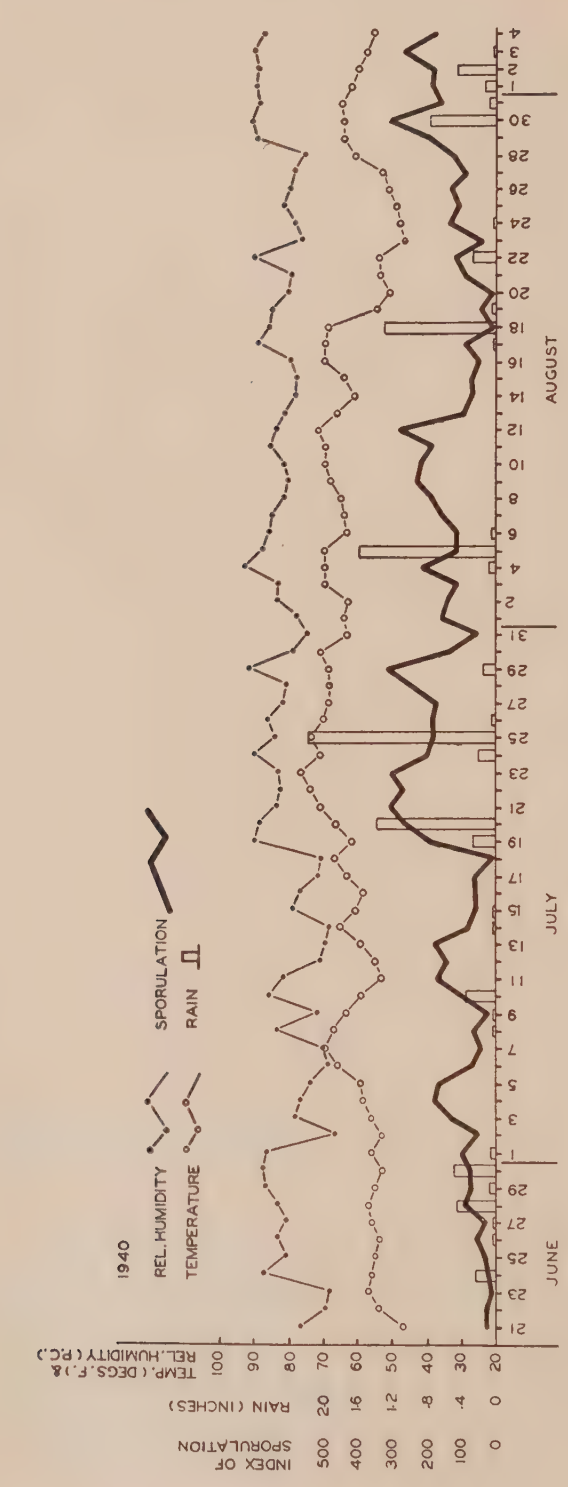


FIG. 16. Daily index of sporulation, daily mean relative humidity, daily mean temperature, and rain, June 21 to September 4, 1940.

tinuously strong to weak sporulation, as indicated by Graph No. 7, and long periods of extremely light activity may, with equal abruptness, end with heavy sporulation (Graph No. 16).

No explanation could be found for the difference in behavior among the individual sporophores. There was no apparent relationship with size of sporophore or position on tree. In some instances strong producers were adjacent to weak ones on the same tree. Fig. 15 shows that the brief periods of inactivity were well distributed among all sporophores, irrespective of rate of sporulation at other times.

Notwithstanding these dissimilar features, the graphs in Fig. 15 indicate a decided tendency toward coincidence of fluctuation in rate of sporulation among the sporophores. The most conspicuous instance of this is the strong sporulation indicated for all sporophores on July 19 and 20. By holding Fig. 15 horizontally at eye-level and sighting over the graphs from the bottom of the figure toward the top, one can readily distinguish several series of corresponding trends in the 18 graphs.

The averages of all observations from June 21 to September 4 are indicated graphically in Fig. 16. In order to study the relation of sporulation to meteorological factors during this period, graphs representing daily mean relative humidity, daily mean temperature, and precipitation are included in the same figure.

Data on relative humidity and temperature were obtained by means of hygrothermographs operated in the immediate vicinity of the sporophore-bearing trees. The daily means were determined by averaging the values indicated by the hygrothermographs at two-hour intervals throughout each 24-hr. period beginning at 8.00 a.m., the time at which the spore traps were changed daily.

The graphs in Fig. 16 indicate that the relationship between sporulation and meteorological factors with respect to seasonal fluctuation is similar to that already demonstrated with respect to daily fluctuation. Here also, it is apparent that sporulation is favored by simultaneously high relative humidity and temperature. This is particularly noticeable in the period from July 19 to August 14 during which there is a strong tendency for the graphs of relative humidity and temperature to follow parallel trends. The corresponding trends in the graph of sporulation are readily apparent here.

However, the graphs also indicate that sporulation is not governed entirely by the two factors thus far considered. It is not known what additional influences are involved, though it appears that precipitation may have an effect not necessarily related to relative humidity. Sporulation sometimes tends to decrease toward the end of prolonged periods without rain, regardless of favorable conditions of relative humidity and temperature. This is illustrated in Fig. 16 where the graph of sporulation descends to an extreme low on August 18 although the graphs of temperature and relative humidity remain high. This relationship is suggested also by the graphs for the period prior to July 19. Similar observations were made also in the following

summer. It is quite possible that rain water may be absorbed by the sporophores or may find its way into the associated rotten wood of the host, and that moisture in this form may influence sporulation strongly at first and to a decreasing degree as it evaporates.

The intermittent observations following September 4 indicated that sporulation may occur at any time during autumn when the maximum temperature of the day is sufficiently high. At this season relative humidity is continuously high and temperature appears to be the major limiting factor. Strong sporulation occurred in temperatures as low as 46° F. The lowest temperature at which sporulation was observed was 40° F. and the latest recorded date of sporulation was November 22.

Observations on favorable days were resumed the following spring on March 27, and daily observations on May 6. The first observed sporulation occurred on April 21 following the first heavy rains of the unusually dry season. This was one of the warmest days of the year to that date, with a maximum temperature of 57° F. By May 5 all five sporophores were sporulating and this continued until observations were discontinued on June 30. During this period the results were similar in every respect to those of the previous year, which adds support to the conclusions already recorded.

Discussion

The results of this study do not indicate any relationship between site and percentage of cubic volume of decay. Eklund and Wennmark (11) found that rapidly growing stands of *Populus tremula* L. in Sweden suffered less from *F. igniarius* decay than did those of slower growth. On the other hand, Schmitz and Jackson (24) in their investigation of *F. igniarius* decay of aspen in Minnesota concluded that rate of growth did not affect the percentage of decay appreciably. They were of the opinion that the problem merited further study.

In a recent study of poplar decay north of Lake Superior, Black (3) found that on a percentage basis cull losses were consistently greater in trees on the poorer sites. This does not necessarily disagree with the results obtained at Petawawa, since Black's conclusion was based on arbitrarily defined cull which includes a considerable proportion of sound wood, while the Petawawa results refer to percentage of actually decayed wood. When decay at Petawawa is expressed in terms of board feet (Table VIII), which also involves arbitrarily defined cull, a higher percentage of cull on the poorer sites appears here also. This is at least partly explainable by the fact that a given percentage of actual decay results in a higher percentage of cull in the smaller trees of poor sites than in the larger trees that occur on good sites, when measured in board feet.

The percentages of decay found by Schmitz and Jackson (24) in Minnesota were considerably higher than those reported here for Petawawa. Their results indicate that "intermediate and final" stage of decay occupies 4.8% of the merchantable volume at 30 years, 11.4% at 50 years, and 20.5% at 70 years. Their combined "intermediate and final" stages of decay correspond

to the stage included in the volumes shown for Petawawa; their definition of merchantable volume, and their methods of field measurements and computation were also practically the same.

That decay conditions at Petawawa differ from those in Minnesota is further suggested by the fact that Brown (6) considered it feasible to establish a "rule of thumb" for estimating cull in aspen stands, based on the diameter of decay one foot from the ground. Such a basis could not be applied in the Petawawa stands, as in many instances the decay does not extend as low as stump height (23). Horton and Hendee (16) state with reference to *F. igniarius* decay in aspen in the Chippewa National Forest of Minnesota, that very often a scaler is confronted with a log that, although perfectly sound on both ends, may be a complete cull. Such logs could only be found where advanced decay occurs without extending as low as stump height. Their findings appear to conform more closely with conditions at Petawawa.

Decay is an important consideration in determining the age at which a given stand should be harvested. In his brief treatment of rotation with respect to disease, Meinecke (19) points out that under North American conditions predetermined rotations are likely to have little more than theoretical value, and that in many cases the expression "actual felling age" should be substituted for "rotation". More recently this subject has been summarized by Boyce (5) who concludes that actually there can be only one rotation, and that this is determined by the interaction of financial, technical, silvicultural, and pathological considerations. He points out that the age at which decay causes consequential loss will be well beyond the actual rotation set by other considerations, except in a few short-lived species such as aspen, which is heavily decayed at an early age. That even aspen is not necessarily an exception is indicated by the results obtained at Petawawa where it has been shown that net periodic increment continues to increase up to the age of at least 70 years. At this age the trees are of good merchantable size for sawlogs or veneer. Stands older than this were not available for study. Meinecke (20) studied decay in aspen up to 130 years old in Utah. His curve of net volume continues to rise at that age, but his curve of net periodic increment flattens out between 80 and 90 years, and subsequently remains practically horizontal. Black (3) concluded that in the region studied by him north of Lake Superior, the maximum net periodic increment on good sites, calculated on the basis of "commercial" cull, occurs at 130 years.

Without consideration of circumstances other than decay, it appears that 70 years is the minimum age at which management plans might provide for the final crop removal at Petawawa when the object is to produce sawlogs or veneer logs. At this age the loss caused by decay on a cubic volume basis amounts to slightly more than 4%, while in terms of board feet the loss is somewhat in excess of 16%. For pulpwood measured on the basis of cubic volume the age of harvesting is less critical than for sawlogs and veneer wood, and economic considerations may justify harvesting at an earlier age.

To those with experience in the cutting of poplar, the estimate of decay losses at Petawawa may appear low. In this connection it is pointed out that the actual losses experienced in practice are undoubtedly higher than indicated here. The board-foot volumes presented in Table VIII were calculated from diagrams of the trees drawn to scale on cross-section paper. By this method the "scaler" has the advantage of knowing in advance the exact position and dimensions of all decay and is thus enabled to measure the maximum yield of each tree. In practice, log cutters usually obtain something less than the maximum volume of sound wood, owing to error in judging the extent of hidden decay. Further, in this study no tree was completely culled that contained an acceptable log length. In woods operations it is common practice to leave standing any tree that shows signs of containing a high proportion of cull, it being considered unprofitable to fell these on the chance of obtaining a single log. This practice sometimes leads to excessive wastage, as was shown by Riley and Bier (23).

A considerable amount of wastage has been observed in logging poplar for use as veneer wood. If a bolt of veneer wood contains a rotten core that does not provide a sufficiently firm grip for the chucks of the veneering machine to turn the bolt against the knife, the entire bolt becomes a loss. Many logs containing large volumes of clear sound wood have been rejected because of a relatively small core of decay (Fig. 6). It is held that such loss should not be charged entirely against the decay, since it appears to be reducible, to a large degree, by improvement in the design of veneering machines.

Loss caused by decay can be reduced to a minimum by refinement of utilization practices. In some operations the wastage of sound wood owing to the presence of decay has been largely eliminated by sorting the wood and marketing it as sawlogs, veneer wood, and pulpwood, according to its condition and size.

A commonly quoted recommendation for the control of heartwood decays generally is that diseased trees be removed in order that they can no longer serve as propagation centers of the respective pathogens. Killing of undesirable species and individuals by means of girdling, in order to make the space occupied by them available for use by more valuable trees, is recognized as sound silvicultural practice (2). This method of eliminating heart-rotted trees raises the objection that, even when dead, they continue to support the disease-producing fungi as saprophytes (25), though for how long this may continue undoubtedly depends on various circumstances. Spaulding (27) examined 1768 hardwood trees of several species two to five years after girdling and found 80 of them bearing living sporophores of five different species of heart-rotting fungi that were established in the interior of the trunks before girdling.

In the Petawawa experiment the number of sporophores was reduced by 87% within six years after girdling. It is considered that this result indicates that girdling is a satisfactorily effective method of eliminating sporophores from a stand. This does not necessarily imply that girdling is economically

practicable or that the practice is an effective means of protecting the stand against new infections. The results of viability tests indicate that spores may retain their viability long enough to cause infection after being carried by winds from sources at considerable distances from the stand that is being protected. Nevertheless, the general recommendation that diseased trees be removed whenever this is economically practicable is indisputably sound.

The inoculation experiments have demonstrated that the presence of normal heartwood is not necessarily a prerequisite of attack by *F. igniarius*, and that this fungus is capable of infecting and causing typical decay in sapwood. Record (22) states that the normal formation of heartwood involves loss of the protoplasmic contents of the cells; hence, heartwood is said to be dead, though it may contain living parenchymatous cells for some time. Heartwood is generally darker in color than sapwood, owing to the presence of gums, resins, and other substances. Büsgen and Münch (9) point out that in the vicinity of wounds sapwood may assume the characteristics of normal heartwood as a pathological phenomenon. From this it could be argued that inoculation of sapwood by means of wounds is somewhat of a paradox, since the act of wounding also induces the transformation of the affected sapwood into heartwood.

In *P. tremuloides* and *P. grandidentata* there is no color distinction between heartwood and sapwood, but in freshly cut living trees these regions may be distinguished by the moist appearance of the latter as compared with the drier heartwood. Healthy poplar trees may contain heartwood at as early an age as 10 years. Several healthy trees of both of the above species were examined in the 10-year-old stand in which the successful inoculations were made. At a height of approximately 2 ft. from the ground most of these trees were between 2 and 3 in. in diameter, and all contained a core of heartwood varying from 0.4 to 0.9 in. in diameter.

The term "parasite" is used here in the broad sense that embraces heart rot fungi. Stevens and Young (28) would restrict the use of the term to refer only to an organism that secures part or all of its food materials from the living matter of its host. Link (18), on the other hand, contends that *Fomes pinicola* may be considered a parasite in that it obtains its food from a living individual, even though it may actually attack only the nonliving tissues of the host. A tree affected by heart rot is unquestionably diseased, whether the rot remains confined to the region normally occupied by heartwood or whether it encroaches upon the sapwood, as sometimes occurs. It seems logical from a practical point of view to consider *F. igniarius* as a parasite.

As compared with other groups of plant pathogens, few heart rot fungi have been definitely proved to be parasitic, even in the broad sense of the term, i.e., in comparatively few instances have they been subjected to Koch's postulates. Only the first rule, that of constant association of the organism with the disease, appears to have been applied with any degree of consistency (17). The inoculation experiments at Petawawa provide the required proof for *F. igniarius*.

Hirt (15) also has reported success in infecting aspen and other hardwood species with three species of heart rot fungi including *F. igniarius*. His method was similar to that used at Petawawa except that he used "agar substratum covered with vigorously growing mycelium" for inoculum, and closed the hole "with a sterilized birch dowel which was immediately brushed with white lead paint". Numerous attempts to infect poplar by the use of mycelium growing on agar were unsuccessful at Petawawa. The use of grafting wax instead of wooden plugs to seal the holes proved unsatisfactory. The wax usually became loose within a year and failed to provide the required protection. Results were the same when the bark was coated with shellac before applying the wax.

It has been demonstrated that sporulation in *F. igniarius* occurs continuously from early spring to late autumn, except for irregular brief periods which do not coincide among different sporophores. The discharge of spores on November 13 and November 22, following periods of winter weather with snow, suggests that there is no definite and final cessation of sporulation at the approach of winter. It is further suggested that sporulation may be resumed at any time upon a return of favorable weather. There appears no reason to doubt that this could occur during extreme midwinter thaws, especially in climates of less rigorous winters than where this work was conducted.

Buller (8) found that sporulation of *F. igniarius* on poplar at Winnipeg lasted about two months (August and September). Verral (29) states that in Minnesota the period of sporulation in 1932 appeared to be limited to two weeks in August. The results of the Petawawa study suggest that the observations of these investigators may have been limited to one or more of the intermittent periods of sporulation that actually continue from early spring to late autumn.

The seasonal period of sporulation does not appear to be associated with any macroscopic changes of the pore surface. Buller (8) states that the seasonal period of sporulation in *F. igniarius* is preceded by the pore surface becoming overgrown with a dark brown coating of new hyphae which produce the new tube layer. Formation of a new tube layer in this manner is common at Petawawa, but its occurrence is quite erratic and does not appear related to seasonal periodicity of sporulation. It did not occur at all on some of the sporophores that were active during the year of observation. On others, the overgrowth developed on only part of the pore surface while the remaining part continued to discharge spores.

In some of the sporophores the pores became stuffed with white mycelium in late July and early August, but sporulation continued, though lightly. The most vigorous sporulation occurred in sporophores that maintained a uniform, dark brown pore surface. Microscopic examinations from time to time during the year indicated that elongation of the pores by means of new growth on the edges of the dissepiments progresses continuously throughout the spring, summer, and fall, and that the white "stuffing" in the base of the pore tends to keep pace with the pore elongation, though it sometimes gains until it becomes visible at the mouth of the pore.

Borisov (4) studied sporulation in *Fomes igniarius* on birch, poplar, and alder in the Leningrad district, U.S.S.R., with results similar in many respects to the results obtained at Petawawa. He describes the same type of fluctuation in sporulation, which he ascribes solely to temperature. He does not mention rain as a possible factor and dismisses relative humidity as having no decisive influence, although he notes that it is closely bound to fluctuations of temperature. He recorded the first seasonal discharge of spores on May 12, 1938, and April 29, 1939, and the final discharge from the birch and alder "forms" on December 10. His "form *tremulae*" ceased sporulating on July 17 and thereafter remained inactive. During the season of sporulation Borisov recorded periods of inactivity lasting from two to several days. He suggests as a probable explanation that these are the periods when growth of new hymenium takes place, but presents no supporting evidence. He found that autumnal discharge of spores ceased when the temperature fell below 5° C. (41°F.), which agrees closely with the corresponding temperature (40° F.) observed at Petawawa.

Acknowledgments

The author gratefully acknowledges the assistance and cooperation that has been generously contributed by many persons in the course of this investigation. At the University of Toronto the late Prof. H. S. Jackson accepted the responsibility of the project as a subject for a thesis, and his advice and criticism was particularly helpful. Prof. T. W. Dwight gave valuable advice regarding the statistical analysis of data. In the Division of Botany and Plant Pathology, Department of Agriculture, Ottawa, the work was facilitated in many ways by Dr. H. T. Güssow, Dr. J. H. Craigie, Mr. A. W. McCallum, and Dr. F. L. Drayton. Valued assistance was rendered by Dr. I. M. Mounce, Dr. M. K. Nobles, Dr. R. Macrae, Dr. J. E. Bier, Dr. A. J. Skolko, Dr. L. T. White, Mr. R. M. Lewis, and Mr. W. B. Denyer. Members of the Forestry Branch, Department of Resources and Development, at the Petawawa Forest Experiment Station cooperated in numerous ways.

To all of the above, and others not mentioned by name, the sincere personal thanks of the author are here expressed.

References

1. Anonymous. Petawawa forest experiment station. Can. Dept. Mines Resources, Forest Service Bull. 1938.
2. Baker, F. S. Theory and practice of silviculture. McGraw-Hill Book Company, Inc., New York and London. 1934.
3. Black, R. L. Interim report on the decay of trembling aspen (*Populus tremuloides* Michx.) in the Upper Pic Region of Northern Ontario. Div. Forest Biol., Sci. Service, Can. Dept. Agr. 1951.
4. Borisov, P. N. Some specific biological properties of *Fomes igniarius* Fr. U.S.S.R. Central Forest Research Inst. Bull. 15. 1940.
5. Boyce, J. S. Forest pathology. 2nd ed. McGraw-Hill Book Company, Inc., New York, Toronto, and London. 1948.
6. Brown, R. M. Statistical analyses for finding a simple method for estimating the percentage heart rot in Minnesota aspen. J. Agr. Research, 49 : 929–942. 1934.

7. Buller, A. H. R. Researches on fungi. I. Longmans, Green and Co., London. 1909.
8. Buller, A. H. R. Researches on fungi. II. Longmans, Green and Co., London. 1922.
9. Büsgen M., and Münch, E. The structure and life of forest trees. 3rd ed. Chapman & Hall, Ltd., London. 1929. *Translated by* T. Thomson.
10. Chapman, H. H. Forest mensuration. John Wiley & Sons, Inc., New York. 1924.
11. Eklund, S., and Wennmark, G. Nagra undersokninger av aspskog. Skogsvards Fören. Tid. 23 : 80–104. 1925.
12. Ezekiel, M. Methods of correlation analysis. John Wiley & Sons, Inc., New York. 1930.
13. Halliday, W. E. D. A forest classification for Canada. Can. Dept. Mines Resources Forest Service Bull. 89. 1937. Reprinted 1950.
14. Heimburger, C. C. Forest type classification and soil investigation at Petawawa during 1934. Can. Dept. Mines & Resources. Unpublished report. 1936.
15. Hirt, R. R. Decay of certain northern hardwoods by *Fomes igniarius*, *Poria obliqua*, and *Polyporus glomeratus*. Phytopathology, 39 : 474–480. 1949.
16. Horton, G. S. and Hendee, C. A study of rot in aspen on the Chippewa National Forest. J. Forestry, 32 : 493–494. 1934.
17. Hubert, E. E. An outline of forest pathology. John Wiley & Sons, Inc., New York. 1931.
18. Link, G. K. K. Etiological phytopathology. Phytopathology, 25 : 843–862. 1933.
19. Meinecke, E. P. Forest pathology in forest regulation. U.S. Dept. Agr. Bull. 275. 1916.
20. Meinecke, E. P. Quaking aspen: a study in applied forest pathology. U.S. Dept. Agr. Tech. Bull. 155. 1929.
21. Nobles, Mildred K. Studies in forest pathology. VI. Identification of cultures of wood-rotting fungi. Can. J. Research, C, 26 : 281–431. 1948.
22. Record, S. J. Identification of the economic woods of the United States. John Wiley & Sons, Inc., New York. 1919.
23. Riley, C. G. and Bier, J. E. Extent of decay in poplar as indicated by the presence of sporophores of the fungus *Fomes igniarius* Linn. Forestry Chronicle, 12 : 249–253. 1936.
24. Schmitz, H. and Jackson, W. R. Heart rot of aspen with special reference to forest management in Minnesota. Univ. Minn. Agr. Expt. Sta. Tech. Bull. 50. 1927.
25. Schrenk, H. von and Spaulding, P. Diseases of deciduous forest trees. U.S. Dept. Agr., Bur. Plant Ind. Bull. 149. 1909.
26. Sisam, J. W. B. Site as a factor in silviculture—its determination with special reference to the use of plant indicators. Can. Dept. Mines Resources, Forest Service Silv. Research Note 54. 1938.
27. Spaulding, P. Persistence of heart-rotting fungi in girdled trees. Phytopathology, 24 : 17–18. 1934.
28. Stevens, F. L. and Young, P. A. On the use of the terms saprophyte and parasite. Phytopathology, 17 : 409–411. 1927.
29. Verral, A. F. Variations in *Fomes igniarius* (L.) Gill. Univ. Minn. Agr. Expt. Sta. Tech. Bull. 117. 1937.
30. Wright, W. G. Statistical methods in forest investigative work. Can. Dept. Interior Forestry Branch Bull. 77. 1925.

STUDIES ON THE WITCHES'-BROOM VIRUS DISEASE OF POTATOES IN BRITISH COLUMBIA[1]

By N. S. Wright[2]

Abstract

Plants of the White Rose and Netted Gem potato varieties naturally or artificially inoculated with the witches'-broom of potato virus expressed symptoms only after the progeny of inoculated plants was grown, but symptoms occurred on the foliage of the X virus-immune potato seedling 41956 within eight weeks after grafting. Tomato and tree tomato served as indicator plants on which two apparent strains of the virus could be distinguished. The first strain caused the symptoms on tomato usually associated with the disease on this suscept, but the second strain caused a disease similar to tomato big bud. Attempts to transmit the virus by means of dodder and insects were unsuccessful. An abrupt cessation of cambial activity and consequent underdevelopment of secondary conductive tissue precede the appearance of disease symptoms on potato.

Introduction

The first record of potato witches'-broom was made by Bisby and Tolaas (2) from Minnesota in 1920, although yellow top degenerate described by Whipple (26) in Montana in 1919 was probably one phase of the same disease. The former name is more descriptive than the latter of the main symptom on potato and has been used consistently in subsequent reports of the disease in North America. A disease known as wilding in the British Isles is considered by Murphy and McKay (21) to be caused by the same virus.

Outbreaks of witches'-broom of potato have been of minor economic importance and have been confined for the most part to areas in western Canada (4, 5, 7, 9, 16, 19) and to areas in the northwestern part of the United States (2, 3, 6, 11, 17, 18, 20) and Alaska (15). In addition, witches'-broom has been reported from Poland (24), Russia (12), China (29), and Australia (1).

Hungerford and Dana (11) gave the first complete account of field symptoms and proved that witches'-broom was carried by tubers. They postulated that the disease was spread during the growing season because of its appearance in partly grown plants, the progeny of which produced typically diseased plants. Transmission of witches'-broom by grafting from diseased potatoes to healthy potatoes and tomatoes was reported by Young (27) in 1927 and by Young and Morris (28) in 1928.

In British Columbia, witches'-broom is economically important in the agricultural areas north of the 51st parallel of latitude. Whenever seed potatoes from these northern areas are grown elsewhere, some witches'-broom may be found in the crop, but further spread does not occur. Northern areas are otherwise well-adapted to seed potato growing because of the scarcity of aphids which transmit the potato leaf roll and mosaic viruses. The usual incidence

[1] *Manuscript received June 6, 1952.*

Contribution No. 1187 from the Division of Botany and Plant Pathology, Science Service, Ottawa. Part of a thesis submitted to the Graduate Division, Northern Section, University of California, in partial fulfillment of the requirements for the Ph.D degree.

[2] *Associate Plant Pathologist, Dominion Laboratory of Plant Pathology, The University of British Columbia, Vancouver, B.C.*

of infection does not exceed 2% but, occasionally, as in 1947, up to 15% of the plants in a field have been diseased. The varieties on which field symptoms have been found are White Rose, Netted Gem, Bliss Triumph, Early Epicure, Warba, Katahdin, Wee McGregor, and Green Mountain.

Field Observations

Symptoms

The first symptoms to appear in potato plants naturally inoculated with the witches'-broom virus are dwarfing, upward rolling, and marginal chlorosis of the upper leaflets. Growth of axillary stems causes a characteristic branching of the tops of infected plants. Stems which grow after symptoms appear are cylindrical rather than angular and have bulbous nodes (Fig. 1). When the first symptoms of infection occur by midseason, growth of normally dormant buds below the ground level results in the development of numerous fine stems with simplified leaves around the base of the infected plant (Fig. 2). Some tubers which appear normal are usually found under such plants, but, in addition, numerous small, pointed, and usually sprouted tubers occur (Fig. 3). When tubers from plants showing primary witches'-broom symptoms are grown, several spindling sprouts appear from most of the buds (Fig. 4) and subsequent branching of the sprouts causes the plants which are produced to have extremely numerous, fine, cylindrical stems (Fig. 7). Plants in advanced stages of infection are distinctly dwarfed and chlorotic. Leaves are simplified and tubers are small and numerous.

Infected Tubers from Symptomless Plants

Prompt roguing of witches'-broom diseased plants from potato fields in north-central British Columbia does not always limit the spread of the causal virus. The extent of spread of the virus in the absence of visibly diseased plants was demonstrated by collecting tubers from symptomless plants in 1946. A single tuber was taken from each of 4000 plants in two fields of the White Rose variety and from each of 3000 plants in two fields of the Netted Gem variety. Samples were taken from groups of 100 plants and labeled so that the location from which each composite sample came could be identified on a map of the fields. The samples were planted in 1947 in tuber units; that is, the tubers were cut into several pieces and all pieces from a single tuber were planted in consecutive order. The plots were examined at intervals during the growing season.

Of the 4000 White Rose units, 142 or 3.55%, were infected with the witches'-broom virus and, of the 3000 Netted Gem units, 13 or 0.43%, were infected. In every case all the plants in an infected unit were diseased. Since all the

Field symptoms of witches'-broom on White Rose potato.

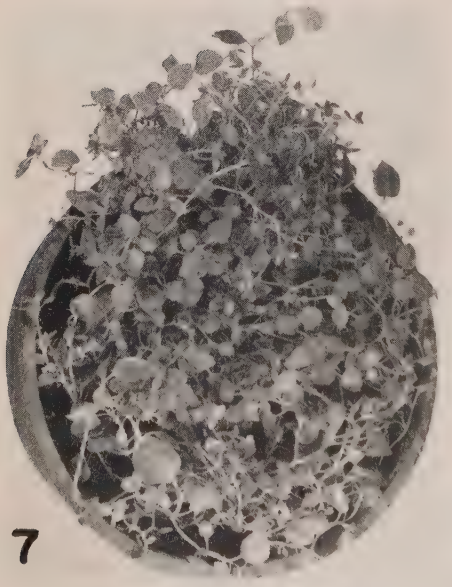
FIG. 1. Dwarfing, upward rolling, and marginal chlorosis of leaflets in early stage of infection. FIG. 2. Numerous, fine stems with simplified leaves around the base of infected plant. FIG. 3. Large number of tubers (93) produced by a single infected plant. FIG. 4. Profuse branching of spindling sprouts on infected potato tuber.


1


2


3


4



Symptoms of witches -broom on artificially inoculated potato seedling 41956.

FIG. 5. Upward rolling and marginal chlorosis of apical leaflets in early stage of infection. Fig. 6. Aerial tubers and numerous fine stems in the axils of leaves of an infected plant. FIG. 7. Extreme fasciculation of stems in an advanced stage of infection. Fig. 8. Simplified leaves and axillary branching of infected stems.

samples had been taken from healthy appearing plants, the conclusion must be drawn that both varieties may carry the witches'-broom virus for at least part of one season without expressing symptoms of infection. There was not a consistent tendency for samples from any particular part of the fields to contain a greater number of diseased plants than samples from other parts of the fields.

Experimental

Transmission to Potato

(*a*) *By tuber plugs:*—Limited transmission to White Rose potato was obtained by the tuber plug method. A hole was made with a $\frac{3}{8}$ in. cork borer in 15 healthy tubers parallel to the long axis and in the region of the vascular ring. This hole was immediately filled with a plug, free of eyes, removed with a $\frac{7}{16}$ in. cork borer from the same region of a witches'-broom infected tuber. The inoculated tubers were then planted. No symptoms occurred on any of the plants produced by the inoculated tubers themselves. However, when the progeny of the inoculated tubers was grown, it was found that four of the 15 original tubers had been infected. No additional infections were found when a sample of tubers from each plant was grown the following year. Check tubers into which virus-free plugs were inserted produced healthy plants throughout.

(*b*) *By stem grafts*:—The witches'-broom virus was transmitted to numerous White Rose and Netted Gem plants by means of cleft and inarch grafts. In all cases it was found that symptoms did not develop until the progeny of the inoculated plants was grown. In order to separate the witches'-broom virus from the potato X virus which is latent in all plants of the above varieties, scions from seven plants of the named varieties were grafted separately to U.S.D.A. potato seedling 41956 which is immune to the potato X virus. Unlike the reaction of the named varieties on which the first symptoms occurred on the progeny of inoculated plants, on seedling 41956 symptoms developed on the grafted plant itself within eight weeks after inoculation.

Although subsequent experiments showed that the viruses obtained from the seven sources were not identical, there was no apparent difference in symptoms on seedling 41956. First symptoms were the upward rolling and marginal chlorosis of apical leaflets, a shortening of the internodes of new growth, and rapid growth of axillary buds (Fig. 5). Aerial tubers and numerous fine stems developed in the axils of leaves (Fig. 6). The normally dormant buds on the tubers below the ground level began to sprout even before the top of the plant had matured and, by the time the top had died, the growth from them had produced the typical or secondary stage of the disease (Fig. 7). There was an apparent loss of the dominance of apical buds and subsequent growth produced an extremely fasciculated haulm. Leaves were smooth, chlorotic, simplified, and distinctly reduced in size (Fig. 8). Large numbers of small tubers were produced.

Transmission to Tomato

Scions from witches'-broom infected potato seedling 41956 were used to transmit virus from the seven sources referred to above to the Bonnie Best variety of tomato. The following technique of grafting was used. Scions were removed from the infected potato plants and a one-inch cut, which sloped upward and inward towards the pith, was made with a sharp razor blade about two inches from the basal end of the scion. A similar cut, which sloped downward, was made in the stock. Scion and stock were then set together and bound with wet raffia. The protruding end of the scion was inserted into a narrow vial which was kept filled with water. The raffia and vial were removed after about 10 days and the part of the scion below the graft was cut off.

Two distinct types of symptoms occurred on the inoculated plants. The first type occurred on plants grafted with scions from six of the seven sources. A cessation of normal apical growth occurred in about five weeks. Terminal leaflets had a purple color and were very dwarfed. Many leaflets and rachises became distorted by a downward cupping and rolling and some had very narrow leaf blades or none at all. The growth of normally dormant buds in the axils of leaves, particularly near the top of the plant, was a consistent symptom. Flowers and fruit were reduced in size but were otherwise normal. A mature plant exhibiting these symptoms is shown in Fig. 9.

Scions from the seventh source transmitted a second and strikingly different type of disease to tomato. This disease, which became evident about six weeks after inoculation, was characterized by etiolated growth and phyllody. Leaf size was reduced but there was no cessation of terminal growth or severe leaf distortion. Growth of axillary stems caused infected plants to have a willowy appearance (Fig. 10). Sepals and petals became leafy structures (Fig. 11) and no fruits were produced.

Transmission to Tree Tomato

Witches'-broom virus from the seven separate sources was transmitted by grafting from potato seedling 41956 to tree tomato (*Cyphomandra betacea* Sendt.), a subtropical solanaceous shrub. Plants were grafted by the method described above for the tomato grafts. Check plants grafted with scions from virus-free potatoes remained healthy (Fig. 12). Two types of symptoms occurred on inoculated plants and this reaction served to separate the viruses into the same two groups as did the reaction of tomato plants. Virus from six of the sources caused the first type of symptoms. Interveinal chlorosis and downward cupping of apical leaves occurred about five weeks after inoculation. Growth of axillary buds near the apex of the stem produced an extreme witches'-broom condition. Leaves on axillary stems were small, mottled, crinkled, and had a tendency to curl downward (Fig. 13).

The second type of symptom, caused by virus from the seventh source, appeared about six weeks after inoculation and was characterized by the growth of stems from axillary buds all along the main stem. Leaves on axillary

Witches'-broom of potato on Bonny Best tomato.

FIG. 9. Mature plant showing the first type of symptoms caused by the witches'-broom virus. Fig. 10. Mature plant showing the second type of symptoms caused by the witches'-broom virus. Fig. 11. Inflorescence with phylloid sepals and petals.

Witches'-broom of potato on tree tomato, *Cyphomandra betacea*.

FIG. 12. Normal plant—check for Figs. 13 and 14. FIG. 13. Tree tomato infected with the first strain of the virus, showing small, mottled, crinkled, and downward curled leaves on axillary stems. Fig. 14. Tree tomato infected with the second strain of the virus, showing smooth, uniformly chlorotic leaves on axillary stems.

branches were smooth, uniformly chlorotic, and reduced somewhat in size (Fig. 14), but there was no mottling, crinkling, or extreme hypotrophy.

Scions from plants with each type of symptom were grafted to several seedlings of tomato and tree tomato and to potato. The two types produced their distinctive symptoms on tomato and tree tomato, but, on potato, there was no evident difference and typical witches'-broom symptoms were produced by virus from each source. These results suggest that the witches'-broom of potato virus exists in more than one strain.

Dodder

Kunkel (13) transmitted a potato witches'-broom virus which he obtained from Maine to potato, *Vinca rosea* L., *Nicotiana rustica* L., tomato, and sugar beet, by means of the dodder, *Cuscuta campestris* Yuncker. A witches'-broom type of disease was produced on the above plants.

Cuscuta campestris that had parasitized potato plants infected with the witches'-broom virus for six to eight weeks was placed on 54 healthy potato plants, 150 tomato plants, 50 *Datura stramonium* L. plants, 50 *Nicotiana tabacum* L. plants, 50 *N. rustica* plants, 50 *N. glutinosa* L. plants, 25 *Solanum melongena* L. plants, 25 *Capsicum annuum* L. plants, 7 sugar beet plants, 29 *Vinca rosea* plants, 50 celery plants, 50 *Plantago major* L. plants, and 50 China aster plants. The dodder was allowed to parasitize the test plants for six weeks after which it was removed and all plants were kept under observation until maturity. In the potato experiments, the second and third generations of tubers were grown. In no case was there any evidence of transmission.

Insects

Attempts were made to effect transmission of the witches'-broom virus by transferring caged colonies of *Macrosteles divisus* (Uhler), *Colladonus geminatus* (Van Duzee), *Circulifer tenellus* (Baker), *Myzus persicae* (Sulzer), and *M. pseudosolani* Theobald from diseased to healthy potatoes. All insects were allowed to feed on infected plants for at least 24 hr. and on test plants for several days. A total of 124 colonies were tested. Because of a possible latent period of unknown duration, most of the colonies were either transferred through a series of healthy plants or retained for several days on an intermediate host prior to testing. To date there has been no evidence of transmission by any of the above insect species.

Histological

Transverse sections from corresponding internodal regions of healthy and diseased potato stems were fixed in Karpechenko's fixing solution (22), dehydrated by the glycerin method (23), and stained by the iron alum-hematoxylin schedule (22). The most evident difference between healthy and diseased tissue was in the amount of conductive tissue formed. There was evidence of a cessation of activity of both fascicular and interfascicular cambium in plants which showed symptoms of witches'-broom.

In a vascular bundle of the stem of healthy potato, a considerable amount of secondary xylem tissue occurs even in sections of the uppermost part of the stem (Fig. 15). Stems of plants in an advanced stage of infection are greatly reduced in size and there is a corresponding reduction in the amount of xylem tissue that occurs in the vascular bundles (Fig. 16). The interfascicular cambium is active in the upper parts of virus-free potato stems (Fig. 17), but the occurrence of thick-walled cells adjacent to the cambium in corresponding sections of plants in early stages of witches'-broom infection (Fig. 18) indicates that in such plants the interfascicular cambium has ceased to function. Further evidence of this cessation of activity is the complete absence of interfascicular cambium in stems of potato plants in advanced stages of infection (Fig. 19).

Discussion

The fact that the White Rose and Netted Gem potato varieties may carry the witches'-broom virus for one growing season without an expression of symptoms may account for the spread of the disease in crops which contain few, if any, visibly infected plants. In such crops the major source of inoculum is probably infected, but as yet symptomless, plants. Although an insect vector has not yet been discovered, the variable incidence of the disease from year to year could be accounted for by assuming a seasonal variation in the numbers and activity of such a vector.

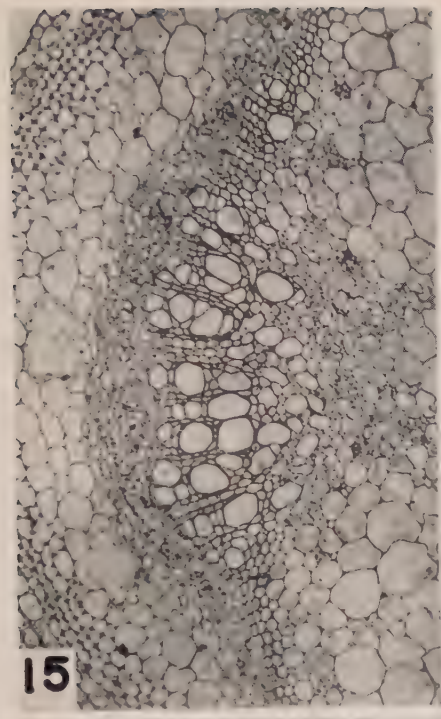
The development of two distinct types of symptoms on tomato and tree tomato by the witches'-broom of potato virus is the first indication that strains of this virus exist. The symptoms caused on tomato by each strain differ so greatly that they would be considered as distinctly different diseases if found under field conditions. Actually, witches'-broom of tomato has not been reported in field plantings.

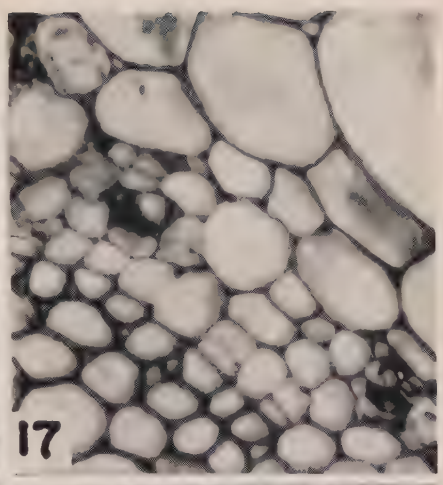
The disease on tomato caused by the first strain of the virus (Fig. 9) resembled the disease transmitted to tomato from witches'-broom infected potato by Young and Morris (28) and by Kunkel (13). The disease caused by the second strain of the virus (Figs. 10 and 11) resembled tomato big bud which has been reported from Australia by Samuel, Bald, and Eardley (25) and from the northwestern part of the United States of America by Dana (8). It is interesting to note that Helson (10) obtained a disease on tomato similar to big bud when he transmitted witches'-broom of alfalfa (lucerne) to tomato by means of the leafhopper *Orosius argentatus* (Evans). The symptoms of big bud and alfalfa witches'-broom were similar in four other suscepts and he

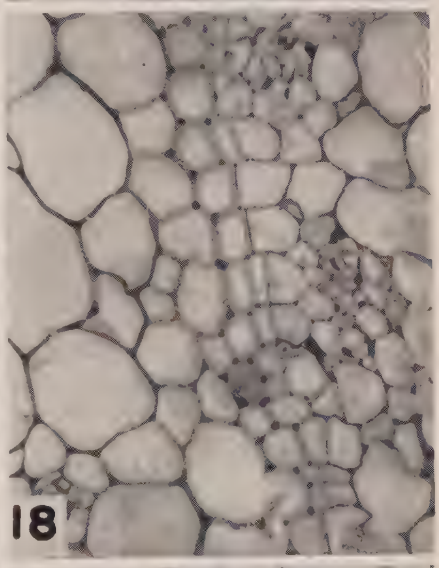
---

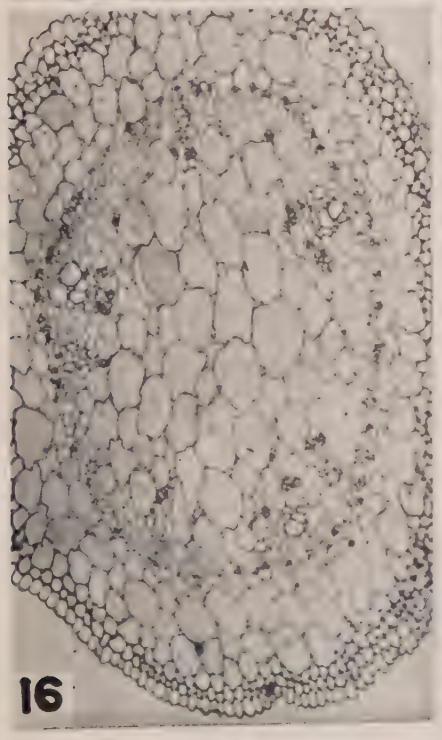
Histological effects of the witches'-broom virus on cambial activity in stems of potato seedling 41956.

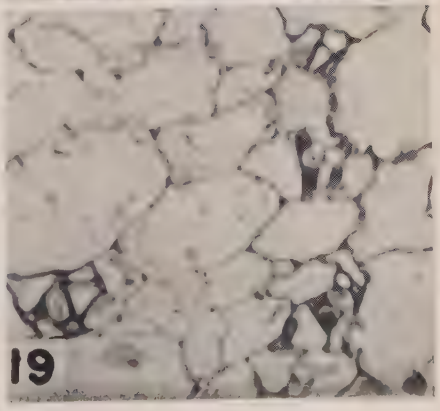
FIG. 15. Normal stem bundle—check for Fig. 16. FIG. 16. Cross section of the stem of a plant in an advanced stage of infection, showing the reduced amount of conductive tissue in vascular bundles. Fig. 17. Normal tissues in the region of the interfascicular cambium—check for Figs. 18 and 19. FIG. 18. Interfascicular cambium region of a plant with early symptoms of infection, showing the occurrence of thick-walled cells adjacent to the cambium. FIG. 19. Interfascicular cambium region of a plant in an advanced stage of infection, showing complete absence of cambium between outer and inner phloem groups. Magnification: Figs. 15 and 16 at 150×; all others at 600×.


15


17


18


16


19

concludes that "the two diseases may be caused by the same virus or by strains of it". Kunkel (14) transmitted alfalfa witches'-broom to potato by means of dodder and obtained a "rather coarse witch's-broom type of growth". His illustration of the disease on potato resembles witches'-broom of potato that occurs in British Columbia. The production of big bud symptoms on tomato by a strain of the witches'-broom of potato virus suggests that this virus may have a strain relationship to the big bud virus which is believed by Helson (10) to have a similar relationship to alfalfa witches'-broom.

Acknowledgments

The writer gratefully acknowledges the guidance given by Dr. M. W. Gardner, Dr. J. W. Oswald, and Dr. T. E. Rawlins, Division of Plant Pathology, and by Dr. J. H. Freitag, Division of Entomology, University of California. Appreciation is also expressed to Messrs. R. Stace-Smith, H. R. MacCarthy J. Bancroft, and R. Hughes for assistance with greenhouse and field plot work at Vancouver.

References

1. Anonymous. Virus diseases of potatoes. Agr. Gaz. N. S. Wales, 54 : 359–362. 1943.
2. Bisby, G. R. and Tolaas, A. G. Potato diseases in Minnesota. Univ. Minn. Agr. Expt. Sta. Bull. 190. 1920.
3. Brentzel, W. E. Types of potato virus diseases in North Dakota. North Dakota Agr. Expt. Sta. Bull. 282. 1935.
4. Conners, I. L. Can. Plant Disease Survey Rept. 11 (1931), p. 48. 1932; 12 (1932), p. 47. 1933; 13 (1933), p. 32. 1934; 15 (1935), p. 36. 1936; 16 (1936), p. 38. 1937; 17 (1937), p. 40. 1938; 18 (1938), p. 49. 1939; 19 (1939), p. 55. 1940; 20 (1940), p. 50. 1941.
5. Conners, I. L. and Savile, D. B. O. Can. Plant Disease Survey Rept. 21 (1941), p. 50. 1942; 22 (1942), p. 61. 1943; 23 (1943), p. 68. 1944; 24 (1944), p. 67. 1945; 25 (1945), p. 74. 1946; 26 (1946), p. 54. 1947; 27 (1947), p. 71. 1948; 28 (1948), p. 63. 1949; 29 (1949), p. 67. 1950; 30 (1950), p. 81. 1951.
6. Coons, G. H. and Kotila, J. E. Michigan potato diseases. Mich. Agr. Expt. Sta. Spec. Bull. 125. 1923.
7. Cutler, G. H. and Sanford, G. B. Potato diseases. Alberta Univ. Coll. Agr. Field Husb. Circ. 7. 1921.
8. Dana, B. F. Occurrence of big bud of tomato in the Pacific Northwest. Phytopathology, 30 : 866–869. 1940.
9. Drayton, F. L. A summary of the prevalence of plant diseases in the Dominion of Canada 1920–1924. Can. Dept. Agr. Bull. 71 (n.s.). 1926.
10. Helson, G. A. H. The transmission of witches' broom virus disease of lucerne by the common brown leafhopper, *Orosius argentatus* (Evans). Australian J. Sci. Research, 4 : 115–124. 1951.
11. Hungerford, Chas. W. and Dana, B. F. Witches' broom of potatoes in the Northwest. Phytopathology, 14 : 372–383. 1924.
12. Jaczewski, A. A. (Witches' brooms of the potato.) Mater. Mikol. i Fitopat. 5 : 117–128. 1926. (In Russian.) *Abstracted in* Rev. Applied Mycol. 6 : 746. 1927.
13. Kunkel, L. O. Potato witches'-broom transmission by dodder and cure by heat. Proc. Am. Phil. Soc. 86 : 470–475. 1943.
14. Kunkel, L. O. Transmission of alfalfa witch's broom to nonleguminous plants by dodder, and cure in periwinkle by heat. Phytopathology, 42 : 27–31. 1952.
15. Lefebvre, C. L. Observations on plant diseases in Alaska. Plant Disease Reptr. 34 : 3–4. 1950.
16. McCurry, J. B. and Hicks, A. J. Annual report on the prevalence of plant diseases in the Dominion of Canada. Rept. 5 (1925) p. 55. 1926; 6 (1926) p. 26. 1927.

17. McKay, M. B. and Dykstra, T. P. Potato diseases in Oregon and their control. Ore. Agr. Expt. Sta. Circ. 96. 1930.
18. McKay, M. B., Dykstra, T. P., Morris, H. E., Young, P. A., Richards, B. L., and Blood, H. L. Virus and viruslike diseases of the potato in the northwest and their control. U.S. Dept. Agr. Circ. 271. 1933.
19. McLarty, H. R. Witches' broom of potatoes. Sci. Agr. 6 : 394–395. 1926.
20. Melhus, I. E., Shepherd, D. R., and Corkle, Marie A. Diseases of potatoes in Iowa. Proc. Iowa Acad Sci. 48 : 133–146. 1941.
21. Murphy, Paul A. and McKay, Robert. A comparison of some European and American virus diseases of the potato. Sci. Proc. Roy. Dublin Soc. 20 : 347–358. 1932.
22. Rawlins, T. E. Phytopathological and botanical research methods. John Wiley & Sons, Inc., New York. 1933.
23. Rawlins, T. E. and Takahashi, William N. Elimination of distortion and poor staining in paraffin sections of plant tissues. Stain Technol. 22 : 99–102. 1947.
24. Ruszkowski, J., Zweigbaumówna, Z., and Blockówna, H. (Diseases of cultivated plants in Poland in 1937.) Roszn. Oehr. Rosl. 5 : 49–102. 1938. *Abstracted in* Rev. Applied Mycol. 18 : 155. 1939.
25. Samuel, Geoffrey, Bald, J. G., and Eardley, C. M. "Big bud", a virus disease of the tomato. Phytopathology, 23 : 641–653. 1933.
26. Whipple, O. B. Degeneration in potatoes. Univ. Mont. Agr. Expt. Sta. Bull. 130. 1919.
27. Young, P. A. Transmission of potato witches' broom to tomatoes and potatoes. Science, 66 (N.S.) : 304–306. 1927.
28. Young, P. A. and Morris, H. E. Witches' broom of potatoes and tomatoes. J. Agr. Research, 36 : 835–854. 1928.
29. Yu, T. F. A list of plant viroses observed in China. Phytopathology, 29 : 459–461. 1939.

RADIATION-INDUCED MUTATIONS IN WHEAT AND BARLEY[1]

By T. J. Arnason[2], C. O. Person[3], and J. M. Naylor[3]

Abstract

The effectiveness of absorbed radiophosphorus for mutation induction in wheat and barley has been studied. The P^{32} was supplied to seedlings and to young plants. Chromosome aberrations found in groups of several cells at meiosis were counted but single cell aberrations were not. Following some dosages of P^{32}, as many as one-third of the treated plants were found to have blocks or clusters of aberrant cells. Samples of R_2 (progeny of treated) plants of vulgare wheat and of common barley were also examined for the presence of chromosome aberrations at meiosis. The samples consisted of 143 wheat and 128 barley plants. Approximately 8 to 19% of wheat and 6 to 11% of barley plants of different treatment groups had aberrations. Phenotypic mutants were found in barley, einkorn, and vulgare wheat. Chlorophyll mutants occurred in all of these though no albinos were produced in *vulgare*. The R_2 and R_3 of *vulgare*, consisting of 10,443 plants from 258 treated R_1 plants, included 15 recognized mutants. The original mutants did not breed true. Offspring of some mutants included only mutant and normal-appearing plants. Other mutants gave a variety of new phenotypic forms; some of these are true-breeding. Nearly all of the wheat mutants gave evidence of chromosome aberrations. Most of the phenotypic changes are therefore attributed to changed gene balance rather than to gene mutation.

A few mutations have also been obtained by irradiating wheat and barley seeds with high-energy X rays from the betatron. Two such mutants in wheat were found to have undergone chromosome breakage and rearrangement.

Introduction

An investigation of the cytological and genetic effects of absorbed radiophosphorus, P^{32}, was begun in 1947. The main object was to add to the fund of knowledge relating to mutagens. In this paper results obtained with wheat and barley are reported. A search for chromosome aberrations was made in R_1 (treated), and R_2 (offspring of treated), plants. Mutant phenotypes were sought and found in R_2 and R_3 populations. Whenever possible the original mutants, their sibs, and their progeny have been investigated cytologically. The reappearance of original mutant phenotypes and the appearance of new ones in offspring of mutants has been noted. Mutations that may be favorable are of special interest to us as they are to students of evolution and especially to those who desire to direct evolution in particular groups of organisms. In wheat or barley, mutations that increase head size, straw diameter, extent of tillering, without any accompanying decrease in fertility, may belong in this category. Mutants having new characters of possible value are objects for special studies which are now under way but are still incomplete.

Plants that have absorbed P^{32} are subjected to low and decreasing intensity of radiations usually for a rather long time. Dosage at low intensity, long-continued, may have genetic and cytological effects somewhat different, at

[1] *Manuscript received February 6, 1952, and, as revised, May 1, 1952. Contribution from the University of Saskatchewan, Saskatoon, Sask.*

[2] *Associate Professor of Biology.*

[3] *Graduate students.*

least in the proportions of gene mutations and chromosomal changes, from those obtained with similar radiation dosages given at a high intensity.

That P^{32} may be absorbed in amounts sufficient to cause considerable chromosome breakage and rearrangement has been indicated in reports from this and other laboratories (1, 2, 3, 4, 5, 9). Some reports (3, 4) indicate that for similar amounts of ionization produced in tissues P^{32} decay may be more effective than X rays. Special P^{32} effects may result from decay of atoms located within meristematic cell nuclei (1). Some changes may owe their origin to atom nucleus recoil and the conversion of a P atom to an S atom at the moment of atom decay.

Studies on mutation induction by high-energy (23 Mev.) X rays produced by the betatron are also under way. Two mutations induced by irradiating moist wheat grains with dosages near 1000 roentgens (r.), are described.

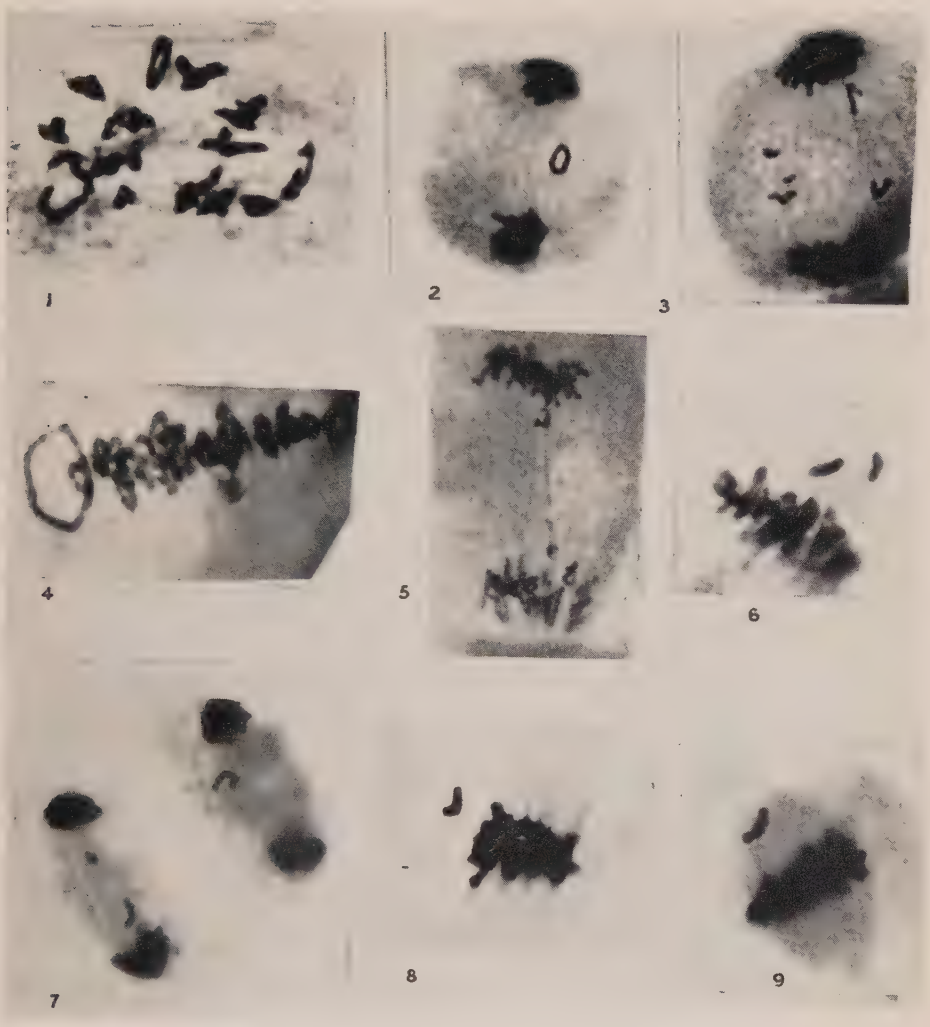
Plants used in the investigation were three species of wheat: *Triticum vulgare Vill.* var. Thatcher, *T. durum* Desf. var. Pelissier, *T. monococcum* L., and barley, *Hordeum vulgare* L. var. Montcalm. A two-rowed barley, variety Hannchen, was used also in early stages of this investigation. Several methods of applying the P^{32} have been described in an earlier report (11). Seeds were either germinated individually in solutions containing measured amounts of P^{32} or the radiophosphorus was added to soil, sand, or solution in which plants were growing. Some plants were treated after the seedling stage was passed.

Chromosome Aberrations in the Treated (R_1) Generation

In untreated plants of the varieties used in this study, examination of some hundreds of anthers has revealed very few aberrations in meiotic cells. Occasionally, at metaphase in durum and vulgare wheats, two univalents occur; still more rarely, split univalents are seen at anaphase. Also, in vulgare wheat the two halves of one bivalent are sometimes not completely separated until anaphase is well advanced, resulting in a bridgelike connection between anaphase groups. In our vulgare variety a structure is often visible at diakinesis that looks like an additional very small bivalent; long slender threads have been seen to connect the structure to a large pair of chromosomes. This structure is therefore considered to be a large satellite.

Treated plants are classed as possessing an aberration if three or more cells of an anther are changed in the same way. In some plants classed as aberrant the aberration is confined to a small group of cells in one anther. At the other extreme the whole spike may be affected. In Table I the observed frequencies of aberrations scored in this way are recorded.

The greatest variety of aberrations appeared in the vulgare wheat with monosomics, translocations, deletions, and inversions all represented. Some of these are illustrated in Figs. 1–3 and 5. In barley and einkorn wheat the most frequently observed aberration figures were of anaphase I and II bridges and fragments. More rarely, translocation complexes were found. Two einkorn plants had patches of pollen mother cells that were tetraploid or near tetraploid with much anaphase irregularity.



FIGS. 1 – 9. Photomicrographs of P^{32}-treated hexaploid wheat microsporocytes. FIG. 1. Metaphase I, ring of four chromosomes and 19 pairs in R_1 plant treated with 6.5 mrd. at the seedling stage. FIG. 2. Telophase I, a ring chromosome lagging. R_1 plant treated with 6.5 mrd. at the seedling stage. FIG. 3. Anaphase I, two lagging, split univalents; one of the split halves of one chromosome is broken. R_1 plant treated with 6.5 mrd. at seedling stage and 65 mrd. at 40 days. FIG. 4. Metaphase I. Ring of four chromosomes. R_2 plant from R_1 treated with 6.5 mrd. at the seedling stage. FIG. 5. Anaphase II, slender bridge. The sister cell appeared normal. R_1 plant treated with 6.5 mrd. at the seedling stage and 65 mrd. at 40 days. FIG. 6. Metaphase I, two univalents. R_3 mutant of mutant club line 79–16. R_1 treatment: 13 mrd. seedling. FIG. 7. Telophase II, long lagging chromosome in one cell, in the sister cell the long chromosome is broken. R_3, large mutant of mutant line 136. R_1 treatment: 13 mrd. seedling. FIG. 8. Metaphase I, univalent, in R_2 plant from R_1 treated with 6.5 mrd. at the seedling stage. FIG. 9. Metaphase I, long univalent in many-tillered, very late maturing large R_4 plant of mutant line 425. R_1 treatment: 6.5 mrd. at 40 days.

FIG. 10. Spikes of mutant line 155. Subcompactoids yield three types of offspring: normal, subcompactoid, and compactoid.

FIG. 11. Spikes of mutant line 266. Monosomics (normal phenotype) at left; presumed nullisomics at right.

TABLE I

THE OBSERVED FREQUENCY OF CHROMOSOME ABERRATIONS IN PLANTS TREATED WITH P^{32}

Treated plants	P^{32}, mrd.[1]	Stage at which P^{32} supplied	No. of plants examined	No. of plants with viable aberrations	Plants aberrant, %
Hordeum vulgare	6.5	Early seedling	35	4	11.4
	65.0	Late (40 days)	44	7	16.0
Triticum monococcum	6.5	Seedling	31	5	16.1
T. durum	6.5	Seedling	21	3	14.3
T. vulgare	6.5	Seedling	28	6	21.4
	6.5	Late (40 days)	8	2	25.0
	65.0	Late (40 days)	17	6	35.3

[1] *Millirutherford; 1 mrd. equals 1000 disintegrations per second.*

Most of the observed aberrations may be directly related to chromosome breakage followed by rearrangement, e.g. translocation rings and inversion bridges with accompanying fragments. Some conditions may be secondary effects, e.g. monosomics may sometimes result from chromosome breakage and loss of both fragments. Sticky chromatin, whether produced by mutation or as a temporary response to radiation, may also result in chromosome duplication or structural change.

Plants heterozygous for particular chromosome changes may produce offspring having other chromosome changes. Some descendants of inversion or translocation heterozygotes may have duplications and deficiencies and offspring of monosomic plants may have telocentric or isochromosomes.

Chromosome Aberrations in the Offspring of Treated Plants (R_2)

Individual R_1 spike progenies were grown in separate rows with, usually, 16 plants in a row. Two or three spikes were collected from each row for cytological examination. The spikes taken may be considered a random sample since the only feature influencing the selection of a head was its stage of development at the time of collection. The object of the sampling was to determine whether any considerable proportion of the R_2 generation was cytologically aberrant. Most of the sample was drawn from hexaploid wheat and diploid barley. As with R_1 material, aberrations were counted only if several or many microsporocytes of a plant had the same aberrations. Usually, in R_2, if an aberration was present it occurred in all the anthers and may be presumed present throughout the plant. However, when P^{32} was supplied late in the development of a plant, some breaks and rearrangements could be expected also in growing embryos, thus producing chimaeras in the R_2 generation. Since the samples examined were not very large and the total

number of aberrations recorded was small it is unlikely that all the types of changes that may occur following P^{32} application are represented. The variety of aberration types observed is sufficient to indicate that any viable rearrangement arising from chromosome breakage may be produced by P^{32} treatment. A list of R_2 aberrations in the several treatment-groups is given in Table II. An R_2 translocation ring of four chromosomes is shown in Fig. 4.

TABLE II

CHROMOSOME ABERRATION FREQUENCY IN SAMPLES OF R_2 PLANTS

Plants	P^{32} supplied to R_1 in days, mrd.			R_2 plants examined	R_2 plants with aberr.		Aberrant features
	0	31	40		No.	%	
T. vulgare	0.65	—	—	39	6	15.4	Anaphase I and II bridges, fragments; univalent
"	6.5	—	—	47	9	19.1	Anaphase bridges; ring of four chromosomes; univ. chromosomes
"	—	—	6.5	12	1	8.3	Unequal pair
"	—	—	65.0	21	4	19.0	Bridges and unequal univalents
"	6.5	6.5	65.0	24	4	16.7	Univalent; anaphase bridges
T. durum	6.5	—	—	12	0	0	
H. vulgare variety Montcalm	6.5	—	—	51	3	5.9	Anaphase bridges and fragments
variety Hannchen	6.5	—	—	41	3	7.3	Anaphase bridges and fragments
"	0.65	—	--	36	4	11.1	Anaphase bridges; ring of four chromosomes

In the samples taken, R_1 plants given small doses of P^{32} contributed, relatively, nearly as many aberrant plants in R_2 as did those receiving larger amounts. The observed frequency of aberrant plants in the different treatment-groups ranged from 8.3–19.1% in vulgare wheat and from 5.9–11.1% in barley. The kind of aberration observed most frequently in barley was anaphase bridges and fragments. As in R_1 the hexaploid wheat displayed a considerable variety including unequal pairs, monosomics, rings of four or six chromosomes, anaphase I and II bridges, and fragments. The greater variety observed in the wheat may simply be related to the larger chromosome numbers giving more chances for the occurrence of simultaneous breaks and rearrangements. A part of the difference may result, however, from survival and multiplication of cells with unbalanced changes in wheat but not in barley.

Mutant Characters

In the R_1 generation, particularly when heavy doses of P^{32} were applied, the leaves were sometimes pale, yellowed, or streaked. In barley a few abnormal spikes were observed, e.g. with very long internodes between the basal sterile spikelet and the second spikelet. In wheat treated at three successive stages of growth extensive yellowing of leaf tips occurred after the last, heavy dose. Seed production was also very low in plants receiving late heavy doses of P^{32}.

In the R_2 generation visible phenotypic effects may be expected if newly arisen recessive alleles are in homozygous condition. Deficiencies and duplications, especially if homozygous, are also likely to cause deviations from the normal phenotype. Inversions and reciprocal translocations are not likely to modify the phenotypes as long as no gene loss nor any change in gene balance accompanies the rearrangement.

Apart from dwarfed plants only chlorophyll mutations were observed in einkorn wheat and in barley. In einkorn wheat a variegated line has been established from a variegated R_2 plant. In barley two color mutations, one albino and one variegated, were found.

The main search for phenotypic changes was, however, concentrated on the *T. vulgare* populations. It became apparent early in the investigation that morphological changes were relatively numerous in this wheat. Some of the new forms appeared to be advantageous, as when spikelet number or amount of tillering was increased. Only one chlorophyll mutation—a variegation—was observed. Characters changed in the several R_2 and R_3 mutants include the following: spike length, spike branching, spikelet number, rachis internode, glume shape, glume texture, awn development, stem diameter, plant height, number of tillers, leaf color, and earliness.

The frequency of the observed character changes in vulgare wheat is as follows:

Plant height—taller 3, dwarfed 4.
Spike length—increased 2, reduced 5.
Head type—branching 1.
Spikelet number—increased 3, reduced 4.
Fertility—sterile 1, reduced 4 plus.
Leaf color—striped, green and gold, 1.
Glume shape—various changes 6.
Awn length—tip-awn to awn, 1.
Grain shape—shortened, broad 1.
Culm diameter—increased 2, decreased 4.
Tillering—increased 4, decreased 5.
Time to mature—increased 6.

In some mutants several characters were altered simultaneously, e.g. in mutant No. 425, plant height, spikelet number, time required to mature; in mutant No. 143-2, head form, fertility, glume shape, amount of tillering, culm

diameter. Segregation in offspring of mutants also adds to the number of changes e.g. the appearance of awns in some offspring of a beardless, short-headed mutant. It is noteworthy that no albinos have appeared in the *vulgare* populations. Seed color changes have not been observed. Data relating to the numbers of mutants in different treatment-groups are presented in Table III. Heads of two of the mutant lines are shown in Figs. 10 and 11, and some meiotic peculiarities of mutants are illustrated in Figs. 6-9. Most of the mutants appeared in progeny of plants receiving high seedling or high late treatments of P^{32}. Apparently none of the original mutants were homozygous. All of them, after presumed selfing (spikes not bagged), have given offspring of diverse phenotypes. The progeny of some mutants have included very few plants that closely resembled the original mutant. In some mutant lines one or more new (mutant) phenotypes have appeared in R_3 or later generations. After 1-3 generations of inbreeding some true-breeding mutant lines have been established.

TABLE III

THE DISTRIBUTION OF PHENOTYPIC MUTANTS AMONG WHEAT LINES VARIOUSLY TREATED WITH P^{32}

Treatment	No. of treated plants	No. of R_1 spikes	No. of R_2 plants	No. of separate mutations	Percentage of treated plants yielding mutants
6.5 mrd. seed and seedling	160	465	7430	2	1.3
13 mrd. seed	50	124	1845	4	8.0
26 mrd seed	8	18	121	1	12.5
6.5 mrd. at 40 days	14	20	173	2	14.3
Multiple and late treatments including 65 mrd. at 40 days	26	87	874	6	23.3

The mutants that have been investigated most fully have all proved to contain some cytological irregularity. Up to the present time information regarding chromosome constitution of the mutants has come solely from direct observations on microsporocytes of mutants, their offspring, and their sibs. Crosses with untreated wheat have been made and later studies of the hybrids may yield useful new information regarding chromosome changes. Testing with nullisomics or monosomics to determine which chromosomes have been altered or lost has not been undertaken.

Information relating to the chromosome condition and the kinds of offspring produced by 13 of the *T. vulgare* mutants has been assembled in summary form in Table IV.

It may be noted that five of the 13 mutants are monosomics. It would appear that the radiation methods used result in a rather high frequency

TABLE IV

SUMMARY OF INFORMATION RELATING TO P^{32}-INDUCED PHENOTYPIC MUTANTS IN VULGARE WHEAT

Ped. No.	Initial P^{32} treatment, mrd.	Mutant phenotype	Offspring of mutant	Associated chromosome aberrations	Probable condition responsible for mutant phenotypes
C297–13	6.5 Seedling	Small, spike, slender. Raised glume shoulder	1 normal 4 mutant	20 II and 1 I	Loss of one chromosome
345	6.5 Seedling	Very tall, late maturing	53 tall 25 normal	20 II and 1 heteromorphic pair	Some normal sibs have translocation ring. Tall, probably heterozygous for duplication and deficiency
79–16	13 Seedling	Club spike	10 normal 6 club	2 univalents	Trisomic or tetrasomic chromosome IX
136	13 Seedling	Large spike, tall plant, large seeds	6 normal 7 mutant	20 II and large I	Absence of one or more growth inhibiting genes
118–13	13 Seedling	6 in. shorter than normal	All short	21 II, one very short	Homozygous deficiency
155	26 Seedling	Compactoid head	15 normal[1] 4 subcompactoid 4 compact	20 II and 2 univalents	Duplication of part of chrom. IX
266	0–0–6.5[2]	Very small head	Not determined	20 II and 1 univalent in normal appearing sib	Loss or one pair of chromosomes
118–6	0–0–65	Speltoid, late	9 speltoid 6 late 1 elong. awns 6 misc. mutant 9 normal	Ring of 4 and univalent	Speltoid deficient for segment of chromosome IX

TABLE IV—*Concluded*

SUMMARY OF INFORMATION RELATING TO P^{32}-INDUCED PHENOTYPIC MUTANTS IN VULGARE WHEAT—*Concluded*

Ped. No.	Initial P^{32} treatment, mrd.	Mutant phenotype	Offspring of mutant	Associated chromosome aberrations	Probable condition responsible for mutant phenotypes
425–16	0–0–6.5	Tall; long heads	5 tall mutant 6 ext. late, tall 2 normal	One univalent	Loss of chromosome
132–3	0–0–65	Leaf stripe, green and gold	22 variegated[3] 2 green	No clear aberration	Gene mutation or small chromosomal change
143–2	6.5–0–65	Large head, high tiller no., late	10 mutant 19 normal	21 II and 1 I or 20 II and 1 III. Sticky chromosomes	Probably mutation to sticky chromosome responsible for chromosome addition
571–1	6.5–0–65	Short head, thick culm	17 thick culm 6 normal	Univalent, sometimes ring-shaped	Altered gene balance
629	6.5–0–65	Mutant glume, club head	Complex segregation for glume size and shape, awns, plant size, earliness	2 univalents	Duplication and deficiency

[1] *In R_5 of this mutant series 16 compactoids yielded 91 compactoids and two doubtful plants, possibly subcompactoid; 18 subcompactoids gave 60 normal, 119 subcompactoids and 38 compactoids; four normal plants produced 58 normal offspring.*

[2] *Numbers refer to treatments at start of germination, at 31 and 40 days respectively.*

[3] *The offspring of three R^3 variegated plants consisted of 38 variegated and 14 normal plants. Nine green sibs (R_4) of variegated plants produced 158 green and no variegated plants.*

of chromosome loss, possibly as a secondary result of simple chromosome breakage. This may be of some interest at the present time since genetic studies in wheat involving monosomics and nullisomics are under way at several stations.

Mutations to give the speltoid and the compactoid (club) types of heads have occurred several times. The speltoids have broad-shouldered, tough, empty glumes; rachis internodes are somewhat lengthened. Some speltoids are bearded in contrast to the tip-awned parent. According to Huskins and Sander (8) and Sears (14) the speltoid complex of characters develops when a deficiency involving the long arm of chromosome IX occurs. The compactoids have shortened rachis and rounded glumes with reduced shoulders. Compactoids may be produced when the long arm of chromosome IX is present in triplicate or quadruplicate. However, genes affecting rachis internode length, beard, and glumes are known to occur also on other chromosomes (14 and 15).

Mutations Induced by High-energy X Rays

Small samples of wheat and barley seeds have been irradiated by high-energy X rays produced by the betatron. The betatron was operated at 23 Mev. which produces radiations at quantum energies from 0 to 23 Mev. The genetic and cytological effects of high-energy radiations may differ in some respects from those of X rays of lower energy (12). Seeds to be irradiated were placed in gelatin capsules which were inserted in cavities in a pressed wood phantom. The beam from the betatron traversed at least 3 cm. of phantom before reaching the seeds. The intensity was about 100 roentgens (r.) per minute as measured by a Victoreen 100-r. condenser chamber placed in the cavity during a preliminary run of the betatron. Different lots of seeds have received doses from 800 r. to 10,060 r.

Of 208 dry vulgare wheat seeds given a dose of 10,060 r. none germinated. Since much larger doses of lower-energy X radiation do not kill all embryos (6), additional tests of killing and seedling-inhibition effects of the betatron radiations are planned. Dry barley seeds have survived doses of 7000 r.

In the progenies of five vulgare wheat plants given seed-treatment of 800 r. one phenotypic mutant appeared. The plant was grassy, many-tillered, late-maturing, and completely sterile. Microsporocytes showed numerous fragments lagging at anaphase 1. It is inferred that multiple breaks and rearrangements occurred.

A single narrow-glumed mutant was also found among the offspring of 16 plants given a seed-treatment of 1200 r. Various new characters appeared in the progeny of this plant including normal glumes, very broad-shouldered glumes, normal and compactoid spike types, and late maturing dwarfs. The original mutant had a ring of six chromosomes at meiosis and it is likely that most of the new characters result from irregular segregation from the translocation complex.

Discussion and Conclusions

It has been shown that absorbed P^{32} present in amounts so small that direct physiological effects are, at most, slight, is nevertheless sufficient to cause many chromosome breaks. The samples of R_1 and R_2 plants taken for cytological analysis indicated that with increase in P^{32} supplied there was an increase in the number of identifiable chromosome "mutations". The samples were too small to show whether aberrations increase linearly with dose. Most of the phenotypic mutants found among progenies of treated plants came from those receiving rather heavy treatments. There are indications also that treatment of plants fairly late in development may be more productive of mutations than early seedling treatments.

Cytological examination of mutants and their sibs and offspring in the hexaploid wheat has shown that most, if not all, of the mutants are characterized by some gross chromosomal change, including chromosome loss and gain and several structural rearrangements. It is therefore considered most likely that these structural changes are causally related to the new morphological or physiological features of the mutant plants and that most of the character changes found in the wheat result from loss or duplication of blocks of genes. Gene mutations may be occurring, possibly even at high rates, without much in the way of observable phenotypic effects because of the presence of duplicate genes.

The length of time broken chromosome ends remain capable of fusion with other broken ends is not certainly known. Estimates based on observations on irradiated *Tradescantia* microspore nuclei range from about one hour (13) to a considerably longer period (10). The simultaneous occurrence of two or more breaks in P^{32} treated nuclei would be expected to be relatively rare. However, several configurations indicating the presence of inversions and translocations have been found. Probably the internally-produced radiations from decaying P^{32} atoms occasionally cause simultaneous multiple breaks.

If a mutation-induction program is to be used in connection with plant breeding the work falls into two distinct parts. The first phase involves the induction and identification of mutations in the selected varieties. In the second phase the promising mutants are tested to determine whether they are of value. Our investigation was concerned with the first phase.

Since mutations are more or less at random, an efficient mutagen is desirable especially if particular kinds of changes are wanted. Various ionizing radiations e.g. X rays and gamma rays are known to be efficient mutagens. That P^{32} also is a satisfactory agent is indicated by the results of some earlier work (1, 3, 4, 5, 9) and by the results described in this paper. The number and variety of mutations induced by P^{32} in our material was, however, much greater in the hexaploid than in the diploid species. The mutagen is, thus, not equally productive of viable mutations in all organisms.

Changed gene balance brought about by imperfect segregation following chromosome breakage is probably responsible for most of the character alter-

ations observed in the treated wheat. It is well known that deficiencies and duplications can be tolerated in polyploids much better than in diploids. In polyploids, therefore, loss or gain of blocks of genes may make significant contributions to the variability of the population. The capacity of vulgare wheat to tolerate such changes and the inability of diploid barley to do so is the most probable reason for the much greater phenotypic diversity in the progenies of treated wheat than in progenies of treated barley.

Some favorable radiation-induced mutations in crop plants have been reported by Gustafsson and his associates (6, 7). Two of the favorable mutations conferred, respectively, increased straw strength and earlier maturity in barley. Of these two characters, the second is favorable only in certain localities, notably those having very short summers. This illustrates the fact that it is not always easy to determine whether a mutant character is advantageous or not. A character conferring an advantage under a particular set of environmental conditions may be less favorable under other conditions. Accordingly, gene combinations selected as desirable for one environmental niche may be discarded as unfit or disadvantageous in others.

Acknowledgments

Preparation of this report has been greatly facilitated by the granting of a sabbatical leave to the senior author. The National Research Council of Canada has aided the investigation by granting substantial financial assistance. Dr. J. W. T. Spinks co-operated closely in the P^{32} treatments and the betatron treatments were supervised by Dr. H. E. Johns. Graduate students G. W. R. Walker, Hulda Regehr, and Elaine Cumming supplied valuable help. We are happy to acknowledge our indebtedness to all the above-mentioned institutions and individuals.

The senior author is grateful to the Genetics Division, University of California, for space and facilities provided during the 1951–52 term. Finally we wish to express our appreciation to Drs. D. R. Cameron, J. A. Jenkins, and A. Vaarama for critical reading of the manuscript.

References

1. Arnason, T. J. Chromosome breakage induced by absorbed radioactive phosphorus. Cold Spring Harbor Symposia Quant. Biol. 13 : 1–5. 1948.
2. Arnason, T. J., Irwin, R. L., and Spinks, J. W. T. Some effects of P^{32} on the development of *Drosophila*. Can. J. Research, D, 27 : 186–194. 1949.
3. Arnason, T. J., Irwin, R. L., and Spinks, J. W. T. P^{32}-induced lethal mutations in *Drosophila*. Can. J. Zool. 29 : 234–239. 1951.
4. Bateman, A. J. and Sinclair, W. K. Mutations induced in *Drosophila* by ingested phosphorus-32. Nature, 165 : 117–118. 1950.
5. Ehrenberg, L., Gustafsson, A., Levan, A., and Wettstein, U. Von. Radiophosphorus, seedling lethality and chromosome disturbances. Hereditas, 35 : 469–484. 1949.
6. Gustafsson, A. Mutations in agricultural plants. Hereditas, 33 : 1–100. 1947.
7. Gustafsson, A. and Nybom, Nils. The viability reaction of some induced and spontaneous mutations in barley. Hereditas, 36 : 113–133. 1950.
8. Huskins, C. Leonard and Sander, Gerhard F. Mutations in polyploid cereals. Can. J. Research, C, 27 : 332–347. 1949.

9. KING, R. C. Genetic changes accompanying the uptake of P^{32} by *Drosophila melanogaster*. Records Genetics Soc. 18 : 98–99. 1949.
10. LANE, G. R. X-ray fractionation and chromosome breakage. Heredity, 5 : 1–35. 1951.
11. NAYLOR, J. M., WALKER, G. W. R., MURRAY, D., PERSON, C. O., SPINKS, J. W. T., and ARNASON, T. J. Uptake of P^{32} by some cereals. Can. J. Botany, 29 : 329–338. 1951.
12. REGEHR, HULDA, ARNASON, T. J., and JOHNS, H. E. Induction of mutation by high-energy X-radiation produced by a betatron. Nature, 166 : 228–229. 1950.
13. SAX, K. An analysis of X-ray induced chromosomal aberrations in *Tradescantia*. Genetics, 25 : 41–68. 1940.
14. SEARS, E. R. Cytogenetic studies with polyploid species of wheat. II. Genetics, 29 : 232–246. 1944.
15. SMITH, S. G., HUSKINS, C. L., and SANDER, G. F. Mutations in polyploid cereals. II. Can. J. Research, C, 27 : 348–393. 1949.

GLUTAMIC CARBOXYLASE OF THE MATURE WHEAT LEAF[1]

By P. Weinberger[2] and K. A. Clendenning[2]

Abstract

A study of the distribution of glutamic carboxylase within the developing wheat plant revealed that it was absent in young plants, and was present only in traces in mature roots, and that it accumulated in mature leaves. Glutamic carboxylase was particularly abundant in the mature and senescent third leaf. Extracts of leaves of other cereals showed only weak activity, while extracts of roots, other than barley, were inactive. The high enzyme activity of the barley root extracts was exceeded only by that of mature wheat leaf extracts. A convenient method is described for enzyme storage at −40° C. and a purification procedure was developed which effected a 500-fold concentration (nitrogen basis). The glutamic carboxylase activity of crude extracts was enhanced by preparatory exposure to phosphate buffer; after selective salt precipitation and lengthy dialysis, activity was reduced, but could be restored by the addition of pyridoxal phosphate. A heat-stable inhibitor of glutamic carboxylase at its pH optimum was found in the ether-soluble organic acid fraction of the cell sap of *Kalanchoe* leaves. Similar inhibiting effects were shown by malate, tartrate, and citrate, but not by succinate, fumarate, aspartate, and alanine. Kinetic studies indicated that the inhibition of plant glutamic carboxylase by cyanide is noncompetitive.

Introduction

Twelve amino acid carboxylases, each of them specific for the natural *l*-isomer of a different amino acid, have been isolated from microorganisms and animal tissues (12). Pyridoxal phosphate functions as the prosthetic group for several if not all of these cyanide-sensitive enzymes (12). Gale (4) has shown that the amino acid carboxylases of bacteria are highly adaptive, and that they are most abundant in old cultures, after cell division has ceased.

Glutamic carboxylase, which specifically decarboxylates *l*(+) glutamic acid, is the only amino acid carboxylase that has yet been isolated from higher plants. It was first demonstrated in clear plant extracts by Schales, Mims, and Schales (9), who showed that pyridoxal phosphate stimulates the dialyzed enzyme and concluded that it serves as the coenzyme (10). Schales and Schales (11) have also employed plant glutamic carboxylase as a specific and quantitative means for determining *l*(+) glutamic acid in protein hydrolyzates. Schales *et al.* (9–11) used carrot roots and squash fruits as sources of glutamic carboxylase, whereas Beevers (1) has recently employed barley roots for this purpose.

In a recent study of the carboxylases of leaves and algae, it was observed that glutamic carboxylase is particularly abundant in wheat leaves, and that it is only weakly reversible (3). The present communication reports an investigation of the distribution of glutamic carboxylase within wheat and other crop plants, as well as studies of its properties. Convenient procedures for its purification and storage are also described.

[1] *Manuscript received May 16, 1952.*

Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa, Ontario. Issued as N.R.C. No. 2847.

[2] *Biochemist, Plant Science Investigations.*

Materials and Methods

Coronation wheat, Nobarb barley, Ajax oats, Redwing flax, Crown millet, and Silverhull buckwheat were grown in greenhouse soil flats and the whole plants were harvested one month from sowing, for the study reported in Table I. Large populations of similarly grown Coronation wheat plants were used in the study of ontogenetic changes in glutamic carboxylase activity reported in Table II. Since it had previously been found that glutamic carboxylase of wheat leaves is located in the supernatant cytoplasmic fraction obtained by high speed centrifugation (20,000 g.) (3), this fraction was used in routine measurements of enzymatic activity as well as in purification studies. Activity measurements were conducted manometrically as previously described (3) and corrected for endogenous carbon dioxide production. (Enzyme in 2 ml. $M/15$ phosphate buffer, pH 5.5; 10 μmoles $l(+)$ glutamate, 30° C., N_2 atmosphere.)

Results

Occurrence in Wheat and Other Crop Plants

Table I shows that glutamic carboxylase is more abundant in wheat leaves than in either wheat roots or in the leaves and roots of other cereal plants of the same age. The glutamic carboxylase activity of the barley root extracts was exceeded only by that of wheat leaf extracts. Weak activity was observed in extracts of oat, millet, and flax leaves, but the corresponding root extracts were inactive. Glutamic carboxylase activity was not observed in extracts of either the leaves or roots of buckwheat plants.

TABLE I

DISTRIBUTION OF GLUTAMIC CARBOXYLASE IN CROP PLANTS

(Plants harvested one month from sowing. Enzyme activities, mm.^{3} CO_2 per hour per milliliter extract)

	Leaf extract	Root extract
Wheat	300	45
Barley	60	120
Oats	45	0
Millet	30	0
Flax	20	0
Buckwheat	0	0

Distribution Within the Developing Wheat Plant

Glutamic carboxylase was not detected in extracts of wheat germ (3) or in leaves of five-day-old wheat seedlings (Table II). The enzyme was present in leaf extracts of 12-day-old seedlings, and its activity increased steadily thereafter in the "tops" with increasing age of the plants, in terms of both extract volume and extract solids. The glutamic carboxylase activity of the first leaf increased until early senescence, but did not attain as high an activity as that of the third leaf (Table II). Activity of the third leaf was much higher than that of the corresponding whole "tops", and increased until the leaves were visibly senescent.

TABLE II

GLUTAMIC CARBOXYLASE IN THE DEVELOPING WHEAT PLANT

Age from sowing, days	Glutamic carboxylase activity ($mm.^3$ CO_2 per hour)					
	"Tops"		First leaf		Third leaf	
	Per ml. supernate	Per 100 mgm. supernatant solids	Per ml. supernate	Per 100 mgm. supernatant solids	Per ml. supernate	Per 100 mgm. supernatant solids
5	Nil	Nil	Nil	Nil	(Unformed)	(Unformed)
12	65	125	65	110	"	"
20	85	130	73	205	535	655
27	120	200	(Withered)	(Withered)	506	(Full sized) 860
32	200	310	"	"	770	1050
40	290	430	"	"	580	1480

Enzyme Storage

After storing wheat leaf extracts in test tubes overnight at −10° C., the glutamic carboxylase activity of the thawed extracts was much lower than that of the extracts before freezing. This loss of enzymatic activity did not occur (Fig. 1) when the extracts were frozen rapidly by the same method as used previously on isolated chloroplasts (2, 5). When the extracts were frozen rapidly in 0.5 *M* sucrose, with the aid of powdered dry ice, and stored immediately at −40° C., the glutamic carboxylase activity of the thawed extracts was higher than that of the original extract even after one year of storage. When the same freezing technique was employed, except that sucrose was omitted, glutamic carboxylase activities were approximately the same before and after freezing. Addition of sucrose to the freshly prepared, unfrozen enzyme extracts resulted in higher enzymatic activity during the first hour at 30° C. (Fig. 1).

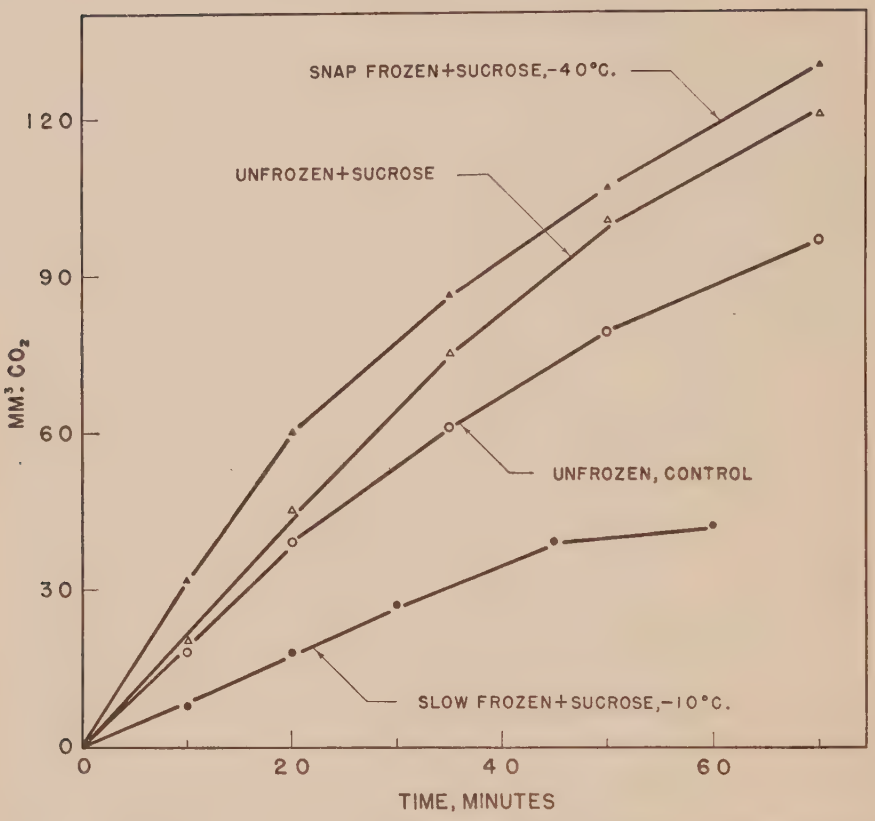


FIG. 1. Effect of sucrose and of freezing treatments on the glutamic carboxylase activity of wheat leaf extracts.

Enzyme Purification

The enzyme extracts used in this study were obtained by high speed centrifugation of the undiluted cell juice. Although impure, these extracts are easily prepared and do not require the addition of synthetic pyridoxal phosphate as coenzyme.

Several techniques were tested as means of increasing the enzyme activity on a solids basis. The following procedure was found to be the most convenient and useful. The chilled wheat leaves were macerated in a mortar with sand or in a meat chopper and the juice was filtered through nylon and centrifuged at 20,000 g. for 15 min. in a cold room (2–5° C.). The chloroplast residue was discarded. The supernatant fraction was then adjusted to pH 4.9 with dilute acetic acid and allowed to stand at 0° C. for 30 min. The sediment obtained by 30 min. centrifugation at 20,000 g. was discarded. Solid ammonium sulphate was added to a concentration of 14% and after one hour at 0° C., the suspension was centrifuged for 15 min. at 20,000 g. and the residue discarded. More ammonium sulphate was added to the supernatant to provide a concentration of 25%, and after one hour at 0° C.

the suspension was centrifuged at 20,000 g. for 15 min. The residue was taken up in a minimum of water, dialyzed free of sulphate against 0.025 *M* phosphate, pH 5.5, and again centrifuged at 20,000 g. The final supernatant fraction required the presence of pyridoxal phosphate (100γ in 2 ml. reaction volume) and had a carboxylase activity which was 1200 times greater on an organic solids basis, and over 500 times greater on a nitrogen basis than that of the original crude leaf extract (Table III).

TABLE III

GLUTAMIC CARBOXYLASE ACTIVITY OF CRUDE AND PURIFIED WHEAT LEAF PREPARATIONS

Sample	Glutamic carboxylase activity (mm.^{3} CO_2 per hour at 30° C.)	
	Per mgm. solids	Per mgm. N
Original crude leaf extract	1.26	23.0
After purification	1500	12,500

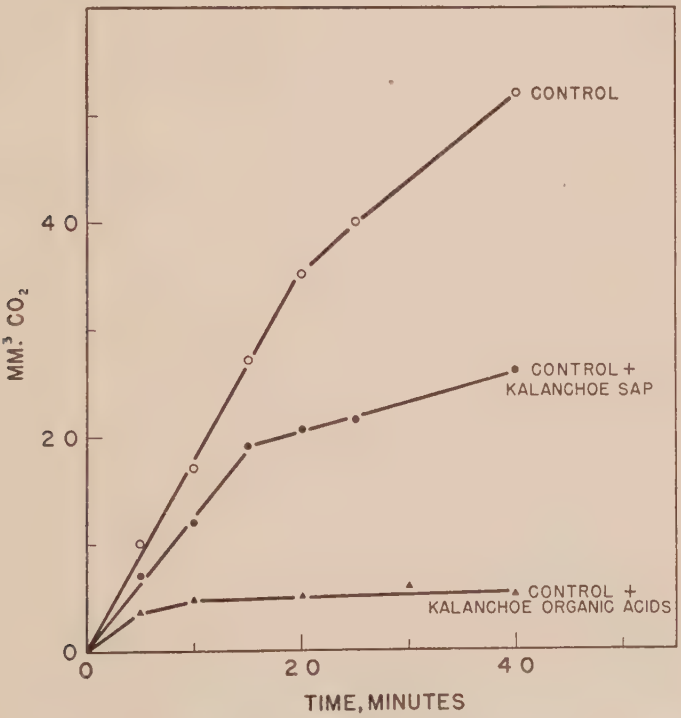


FIG. 2. Inhibition of glutamic carboxylase at its pH optimum (5.5) by *Kalanchoe* leaf sap (1 ml. *Kalanchoe* leaf sap in 2 ml. reaction volume), and by an equivalent volume of *Kalanchoe* ether-soluble organic acids.

Organic Inhibitors of Glutamic Carboxylase

A number of leaf extracts with negligible glutamic carboxylase activity were tested for natural inhibitors by admixture with active wheat leaf extracts. A potent inhibitor was detected in this manner in extracts of the *Kalanchoe* leaf (Fig. 2). Approximately 50% inhibition was observed when 1 ml. of undiluted *Kalanchoe* leaf sap was included in the usual 2 ml. reaction mixture. Since the leaf sap had been adjusted to the pH optimum (5.5) prior to its addition to the glutamic carboxylase system, its inhibiting action was not due to a shift in pH. This natural inhibitor was completely heat-stable and was retained in the cell sap after the coagulated cytoplasmic proteins and chloroplast fragments had been removed.

It had been observed in another connection that similarly neutralized malate solutions (pH 5.5) had a strong inhibiting effect on wheat glutamic carboxylase. The leaves of succulent plants, such as *Kalanchoe* and *Bryophyllum*, contain relatively large amounts of ether-soluble organic acids. Pucher *et al.* (8) have shown that malic and isocitric acids are the principal

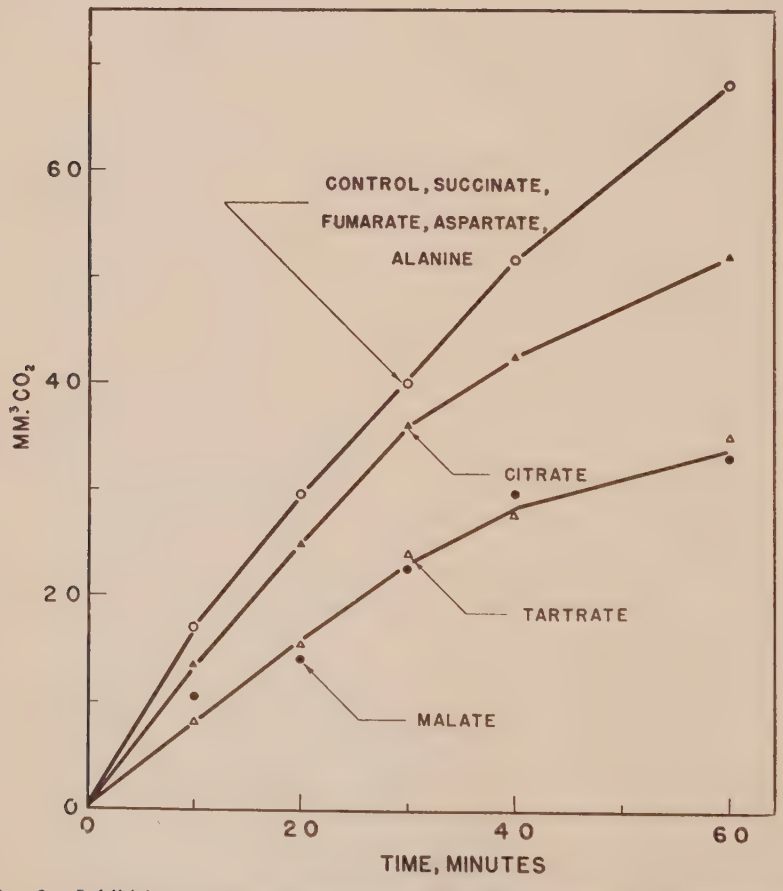


FIG. 3. Inhibition of glutamic carboxylase at its pH optimum (5.5) by organic acids.

organic acids of *Bryophyllum*. The ether-soluble organic acid fraction was therefore isolated from the solids in 10 ml. *Kalanchoe* leaf sap by the method of Pucher, Vickery, and Wakeman (8). After removing the ether on the steam bath, the acids were dissolved in water, neutralized to pH 5.5, and adjusted to 5.0 ml. volume. The inhibiting effect of an equivalent volume of this organic acid solution on wheat glutamic carboxylase was actually stronger than that of the crude *Kalanchoe* leaf sap (Fig. 2).

Malic acid and tartaric acid (0.01 *M*, pH 5.5) both have strong inhibiting effects on glutamic carboxylase (Fig. 3). Pyruvate also inhibits purified glutamic carboxylase derived from wheat leaves. Citrate is also inhibitory although its effect is not as pronounced as that of malate and tartrate under the same conditions. No inhibition was observed with succinate, fumarate, aspartate, and alanine (Fig. 3). The organic acids which inhibit glutamic carboxylase thus have hydroxyl substituents on γ carbons adjacent to terminal carboxyl groups. Related acids which do not inhibit glutamic carboxylase do not possess this molecular configuration.

Kinetic Studies of Glutamic Carboxylase

Schales and Schales (11) found that the substrate concentration at which carrot root glutamic carboxylase attains half maximal speed at its pH optimum,

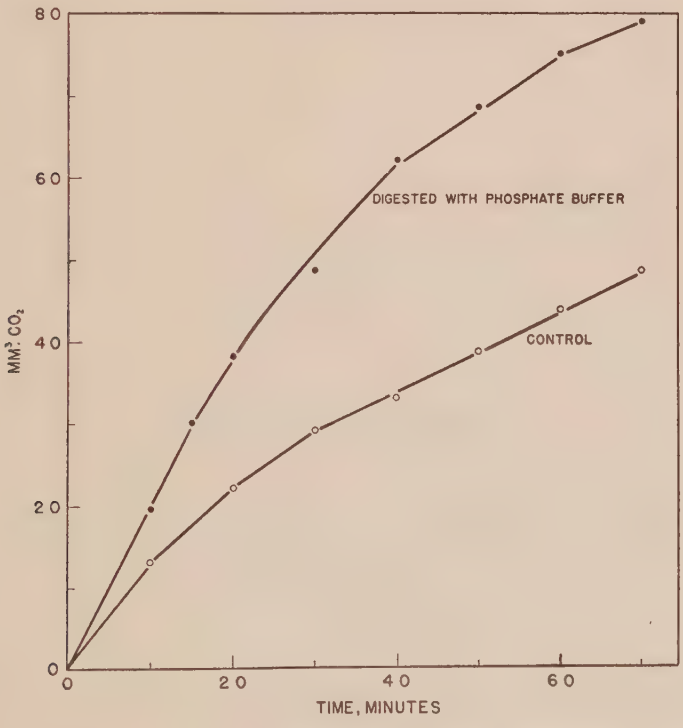


FIG. 4. Stimulation of glutamic carboxylase by 30 min. preparatory digestion with *M*/15 phosphate buffer at 5° C. Control extract received phosphate buffer immediately before equilibration.

is $3.6 \pm 0.4 \times 10^{-3}$ moles per liter. A similar K_s value was observed (3.3×10^{-3} moles per liter) for glutamic carboxylase of the wheat leaf on plotting the reciprocals of the initial velocities against the reciprocals of the corresponding substrate concentrations.

Glutamic carboxylase activity of the wheat leaf is enhanced at 30° C. following incubation at 5° C. for 30 min. with $M/15$ phosphate buffer, pH 5.5 (Fig. 4).

Plant glutamic carboxylase is strongly inhibited by 10^{-4}–10^{-5} M cyanide (6). Kinetic analysis of the decarboxylation reaction in the presence of cyanide is reported in Fig. 5. Employing a constant amount of enzyme in 10^{-4} M cyanide, the degree of inhibition was not reduced on raising the glutamate concentration from 2.5×10^{-3} to 10×10^{-3} M. This observation shows that cyanide is a noncompetitive inhibitor of plant glutamic carboxylase.

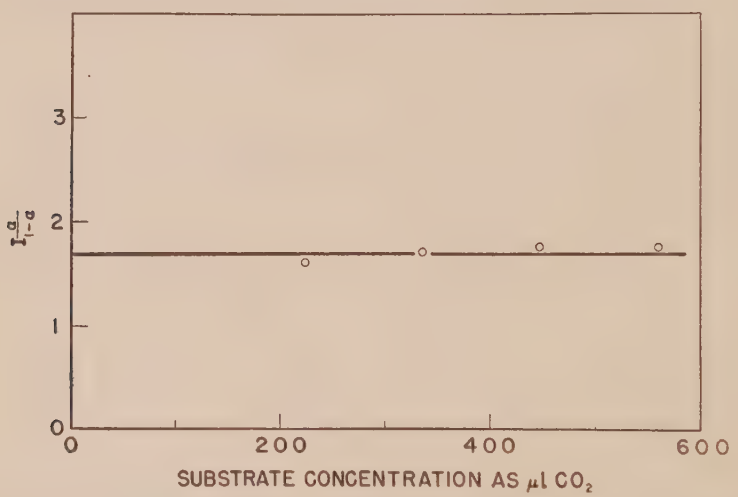


FIG. 5. Noncompetitive inhibition of glutamic carboxylase by 1×10^{-4} M cyanide. I = Concentration of inhibitor, α = fractional activity, v_i/v.

Discussion and Summary

Amino acid carboxylases of bacteria are most abundant in old cultures (4, 12). Glutamic carboxylase activity of higher plants shows a similar relation to the age of certain tissues: this enzyme is more abundant in the cytoplasm of old than of young wheat roots and leaves, and Schales (9) has previously observed that glutamic carboxylase activity is higher in extracts of stored than of freshly harvested carrots. Amino acid carboxylases are adaptive enzymes (4, 12) and the changes in the glutamic acid carboxylase activity of higher plants with age may be associated with changes in the concentration of free glutamic acid.

Mature and senescent third leaves of wheat are a much richer source of glutamic carboxylase than the carrot roots and squash fruits used in earlier

studies (7, 11). Although Beevers (1) has reported as high glutamic carboxylase activity in barley roots as was observed in the present study of wheat leaves, we have not obtained this result with locally grown barley. The mature and senescent leaves of wheat are therefore judged to be one of the richest and most convenient sources of this enzyme.

Schales and Schales (10) have previously reported a K_s of 3.6×10^{-5} moles per liter for carrot root carboxylase and observed that enzymatic activity at 37° C. remained high in the presence of pyridoxal phosphate. Properties similar to those of the wheat enzyme are also shown with respect to pH and inhibition by hydroxylamine (10).

Glutamic carboxylase preparations of much higher purity than those employed previously may readily be prepared from mature wheat leaves, and may be preserved either by lyophilization (11) or by the low temperature storage technique described. The inhibiting effect of α-hydroxy organic acids on this enzyme at its pH optimum is judged worthy of further study.

References

1. Beevers, H. An *l*+ glutamic carboxylase from barley. Biochem. J. 48(2) : 132–137. 1951.
2. Clendenning, K. A. and Gorham, P. R. Photochemical activity of isolated chloroplasts in relation to their source and previous history. Can. J. Research, C, 28 : 114–139. 1950.
3. Clendenning, K. A., Waygood, E. R., and Weinberger, P. The carboxylases of leaves, and their role in photosynthesis. Can. J. Botany, 30 : 395–409. 1952.
4. Gale, E. F. The bacterial amino acid decarboxylases. Advances in Enzymol. 6 : 1–32. 1946.
5. Gorham, P. R. and Clendenning, K. A. Storage of isolated chloroplasts without loss of photochemical activity. Can. J. Research, C, 28 : 513–524. 1950.
6. Okunuki, K. The active group of glutamic carboxylase and its inhibitors. Acta Phytochim. (Japan), 13 : 155–159. 1943.
7. Pucher, G. W., Leavenworth, C. S., Ginter, W. D., and Vickery, H. B. Studies in the metabolism of Crassulacean plants: change in the composition of *Bryophyllum calycinum* during growth. Plant Physiol. 22 : 1–19. 1947.
8. Pucher, G. W., Vickery, H. B., and Wakeman, A. J. Determination of the acids of plant tissue. II. Total organic acids of tobacco leaf. Ind. Eng. Chem. Anal. Ed. 6:140-143. 1943.
9. Schales, O., Mims, V., and Schales, S. S. Glutamic acid decarboxylase of higher plants. I. Distribution, preparation of a clear solution, and nature of the prosthetic group. Arch. Biochem. 10 : 455–465. 1946.
10. Schales, O. and Schales, S. S. Glutamic acid decarboxylase of higher plants. II. pH activity curve, reaction kinetics, and inhibition by hydroxylamine. Arch. Biochem. 11 : 155–166. 1946.
11. Schales, O. and Schales, S. S. Glutamic acid decarboxylase of higher plants. III. Enzymatic determination of *l*(+) glutamic acid. Arch. Biochem. 11 : 445–450. 1946.
12. Sumner, J. B. and Myrback, K. The enzymes. Vol. 1, Part I; Vol. 2, Part I. Academic Press, Inc., New York. 1951.

STUDIES OF CANADIAN THELEPHORACEAE

IX. A CULTURAL AND TAXONOMIC STUDY OF THREE SPECIES OF *PENIOPHORA*[1]

By Constance G. McKeen[2]

Abstract

Close relationship between *Peniophora heterocystidia Burt, P. mutata* (Peck) Höhn. & Litsch., and *P. populnea* (Peck) Burt is indicated by similarities in their fruit bodies, but specific separation is warranted by differences in color and thickness of fruit bodies and in shape and relative numbers of cystidia and gloeocystidia. In addition, *P. populnea* is restricted to the host genus *Populus* and *P. heterocystidia* to broad-leaved trees other than *Populus*. This separation is confirmed by recognizable differences in the cultural characters of the three species and by interfertility tests. Pairings between monosporous mycelia from individual fruit bodies have shown that all three species are hermaphroditic, self-sterile, and interfertile, and of the bipolar type of interfertility. Pairings between monosporous mycelia from fruit bodies of the same species have shown complete or partial fertility, while pairings between fruit bodies of the different species have demonstrated complete sterility. Cultures from fruit bodies typical of *P. allescheri* (Bres.) Sacc. & Syd. behaved like those from typical *P. mutata*, confirming the combination of the two species. In interfertility tests between cultures of *P. mutata*, isolates from *Populus* were completely interfertile, as were those from hosts other than *Populus*, but pairings between isolates from *Populus* and other hosts exhibited various degrees of incompatibility. Sizes of basidiospores and conidia in collections and cultures of *P. mutata* from *Populus* were slightly larger than in collections from other hosts, but differences were not considered sufficiently great to justify the establishment of a variety. Conidiophores and conidia are produced abundantly in cultures of all the species but were found in nature only in two collections of *P. heterocystidia*.

Introduction

The family Thelephoraceae includes groups of species in which gross morphological differentiation is so slight that the separation of species must be made largely on the basis of microscopic characters. To a considerable degree the choice of characters on which separation of species is based has been arbitrary, and may require revision as knowledge of the relationships within the group is increased. In addition, experience has shown that as more specimens of a species are examined, greater variation of certain characters may be found so that, among related forms, no sharp line between species may be evident. In such cases the question may arise as to whether specific entities are recognizable, or whether one species with a wide range of characters is represented. The value of cultural and interfertility studies in providing additional criteria for the delimitation of such closely related species has been demonstrated by

[1] *Manuscript received July 9, 1952.*

Contribution from the Department of Botany, University of Toronto, Toronto, Ont., and Contribution No. 1191 from the Division of Botany and Plant Pathology, Science Service, Department of Agriculture, Ottawa, Canada. Based on a thesis presented in May, 1948, to the University of Toronto in partial fulfillment of the requirements for the degree of Master of Arts.

Previous numbers in this series were published in Can. J. Research, C, 26: 129–139; 143–157. 1948; in C, 27: 147–156; 241 252. 1949; in C, 28: 63–77; 525–534; 716–725. 1950; and in Can. J. Botany, 29: 279–296. 1951.

[2] *Formerly Assistant Mycologist, Central Laboratory, Ottawa, Ontario. This posthumous paper, in preparation at the time of the death of Miss McKeen, has been revised slightly by Mildred K. Nobles in order to incorporate certain suggestions made by the late Prof. H. S. Jackson.*

the work of Mounce and Macrae (15) on *Fomes roseus* (Alb. & Schw.) Cooke and *F. subroseus* (Weir) Overh., of Biggs (2) on *Corticium coronilla* Höhn. & Litsch., of Nobles (19) on the *Trametes serialis* complex, and of others.

The present study was suggested by Prof. H. S. Jackson who had noted the similarities of the microscopic characters of *Peniophora allescheri* (Bres.) Sacc. & Syd., *P. heterocystidia* Burt, *P. mutata* (Peck) Höhn. & Litsch., and *P. populnea* (Peck) Burt. At his request Dr. M. K. Nobles had grown these species in culture and had found that each produced conidia on oedocephaloid heads. As a result of an examination of the type material of *P. allescheri* and authentic material of *P. mutata*, Rogers and Jackson (24) combined the two species, since the morphological characters did not appear to be significantly different. However, it was felt that a thorough comparative study of the morphological characters of the fruit bodies combined with a study of cultures and of interfertility reactions between monosporous mycelia would be advisable in order to establish their relationships clearly. Accordingly, such an investigation was undertaken and, as a result, the validity of the species *P. mutata*, *P. heterocystidia*, and *P. populnea* has been confirmed, the descriptions of their fruit bodies have been revised, and the reduction of *P. allescheri* to synonymy under *P. mutata* has been corroborated.

Materials and Methods

The fruit bodies that were examined and used as the basis for the revised descriptions are listed following the description of each species. At the beginning of the study the specimens were assigned to the different species on the basis of the morphological characters of the fruit bodies. From fresh collections, polysporous and monosporous cultures were isolated and used for comparative cultural studies and interfertility tests. All of the collections are deposited in the Mycological Herbarium, Department of Agriculture, Ottawa, or in the University of Toronto Herbarium. Those collections from which cultures were obtained are listed in Table III.

In describing the appearance of sporophores, the color has been denoted according to the Munsell Book of Color (17). Morphological examinations were made from freehand sections and crushed material, mounted in a 7% aqueous solution of potassium hydroxide and stained with an aqueous solution of phloxine. All measurements were made under the oil immersion lens, those given for cystidia, gloeocystidia, and basidia representing the length from the apex to the septum at the base and the width at the largest diameter, exclusive of sterigmata and incrustation. Basidiospores that were lying flat with the apiculus in view were measured, 30, or occasionally 20, spores being measured from each collection. The range in size is given, followed by the mean in brackets.

Polysporous and monosporous cultures were obtained by sowing basidiospores from a spore deposit on plates of cooled malt extract agar and allowing them to germinate. After 24 to 48 hr., several germinated spores were transferred to tubes of malt agar for polysporous cultures or one for each mono-

sporous culture. To determine the cultural characters, cultures were grown on 2% Difco malt agar in Petri dishes and on malt agar containing gallic or tannic acid, according to the method described by Nobles (20). Microscopic examinations were made at suitable intervals, a portion of the mycelium being crushed in phloxine and potassium hydroxide solution.

Morphological Studies

Peniophora heterocystidia Burt, Ann. Missouri Botan. Garden, 12: 293. 1925. *Peniophora kauffmanii* Burt, Ann. Missouri Botan. Garden, 12: 296. 1925.

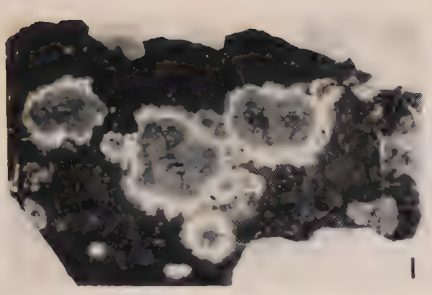
Fructifications (Plate I, Figs. 1 to 4) resupinate, effused, membranaceous, separable from the substratum when moistened, when dry somewhat cracked and buff to pinkish drab in color, 5.0YR7/2 to 10.OYR8/4, margin thin and white. Section as described by Burt, "200–400μ thick, 2-layered, the layer next to substratum usually broad, composed of loosely interwoven, somewhat ascending or longitudinally arranged, hyaline, nodose-septate hyphae 3–4$\frac{1}{2}\mu$ in diameter, the hymenial layer 40–80μ thick." Cystidia present throughout the hymenial layer but not protruding, 25.0–55.0 × 5.0–9.0μ, nodose-septate at the base, obclavate, contents lightly stained in phloxine, walls thick and refractive, the thickening usually greatest in a region 7.0–10.0μ behind the apex, heavily incrusted in a tunicate manner, in aqueous potassium hydroxide the incrustation often splitting longitudinally and floating away from the cystidium (Plate II, Fig. 3); large compact masses of crystals 40.0–100.0 × 20.0–70.0μ present in outer portion of section; gloeocystidia numerous, 40.0–100.0 × 6.0–10.0μ, subtended by a clamp connection, irregularly cylindrical, flexuous, contents staining deeply in phloxine, extending between basidia and occasionally protruding up to 15.0μ (Plate II, Fig. 1); basidia 15.0–30.0 × 5.0–6.0μ, more or less urnshaped, nodose-septate at the base, bearing four slender, tapering sterigmata up to 6.3μ in length (Plate II, Fig. 5); basidiospores hyaline, cylindrical, inner side usually flattened or indented, apiculate, 11.0–15.0 × 3.5–4.5(12.2 × 3.8)μ (Plate II, Fig. 7).

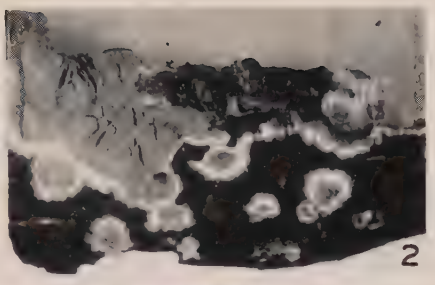
Specimens Examined[1]:

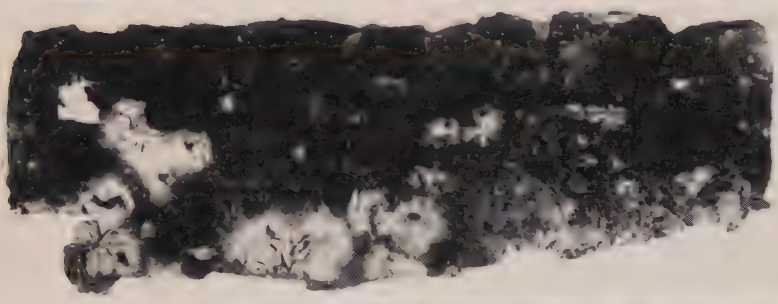
Ontario: on *Acer saccharum*, Dorset, Nov. 13, 1948, V. J. Nordin, DAOM *21308;* on *Acer*, Ottawa, Sept. 16, 1897, J. Macoun, DAOM *F2793*; on *Carpinus caroliniana*, Don Valley near Sunnybrook Park, Toronto, Sept. 12, 1942,

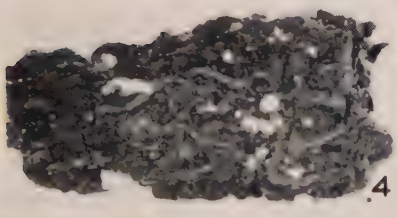
[1] *All collections listed are represented in the Mycological Herbarium of the Department of Agriculture, Ottawa, or in the Mycological Herbarium of the University of Toronto. Symbols preceding the numbers are abbreviations proposed by Lanjouw and Stafleu (12) for the herbaria. In the Ottawa herbarium all collections are listed under its number (DAOM), in the Toronto herbarium, Ontario collections are listed under its number (TRT), parts of collections from other herbaria appear under their original number.*

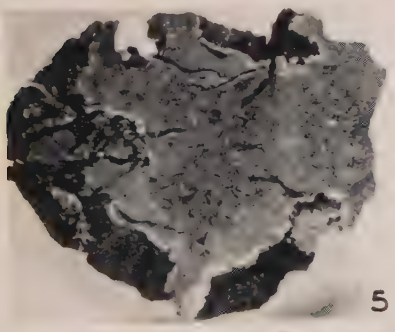
FIGS. 1 to 9. Fruit bodies.
FIGS. 1 to 4. *Peniophora heterocystidia.* FIGS. 1 to 3. Collection DAOM *22547.*
FIG. 4. Collection DAOM *21301,* bearing basidia and conidiophores.
FIGS. 5 and 6. *Peniophora populnea,* collection DAOM *10740.*
FIG. 7. *Peniophora heterocystidia,* DAOM 21308, and *P. mutata,* DAOM *21309.*
FIGS. 8 and 9. *Peniophora mutata,* collection DAOM *21621.*

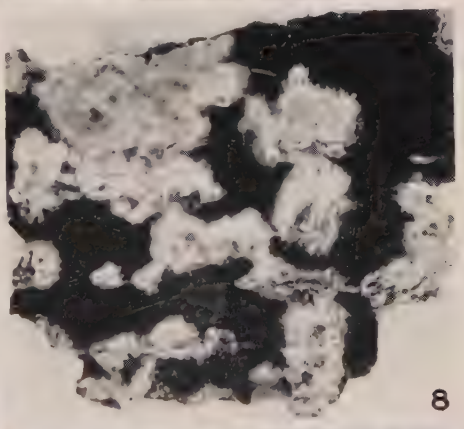

1


2

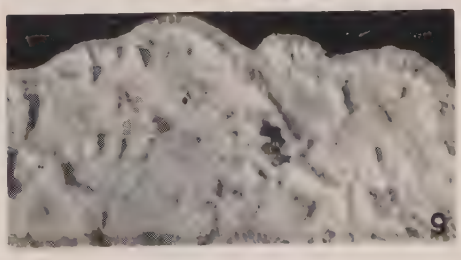

3


4


5


8


6


9

H. S. Jackson, DAOM *10899* (TRT *17578*); Sept. 26, 1946, H. S. J., DAOM *17216* (TRT *21272*); on *Fagus grandifolia*, north of Bolton, Peel Co., Oct. 16, 1948, R. F. Cain, DAOM *21301*; host unknown, near Ottawa, Oct. 9, 1950, C. G. McKeen, DAOM *22547*.

Quebec: on *Acer saccharum*, Chelsea, Oct. 8, 1936, I. Mounce, DAOM *F7228*; on *Acer*, Oct. 9, 1936, M. K. Nobles, DAOM *F7233*; Oct. 8, 1936, J. W. Groves, DAOM *F7234*; Gatineau Park, Oct. 26, 1947, C. G. McKeen, DAOM *17587*; on *Fagus grandifolia*, Buckingham, Sept. 24, 1903, J. Macoun, DAOM *F6260*; host unknown, Gatineau Park, Sept. 25, 1947, C. G. McKeen, DAOM *17551*.

Kentucky: on *Fagus*, Harlan, Sept. 1, 1916, C. H. Kauffman, MO *22827* (in TRT) (type of *P. kauffmanii*).

Michigan: on *Carpinus caroliniana*, La Peer, Oct. 12, 1947, H. S. Jackson, DAOM *17568* (in TRT).

New York: on *Robinia pseudoacacia*, New Windsor, Sept. 30, 1933, H. M. Aloney, BPI *81279* (in TRT).

Pennsylvania: on *Juglans cinerea*, Ross Run, Huntingdon Co., Nov. 19, 1932, L. O. Overholts, DAOM *F3040;* host unknown, Pocono Lake, Monroe Co., July 7, 1933, L. O. O. and G. L. Zundel, DAOM *F4378* (L. O. O. *16777*).

Peniophora heterocystidia was established by Burt (7), who distinguished it "from our other separable species by having incrusted cystidia of the usual size, other very large cystidia up to 20–50μ in diameter, and gloeocystidia". He stated that *P. kauffmanii* Burt differs only in that the "very large cystidia" are lacking. Rogers and Jackson (24) who examined other portions of the type of *P. kauffmanii* found that "large cystidia (or compact aggregations of crystals) - - - - - - are clearly present in certain areas. There are no other differences, and the species are synonymous. *P. heterocystidia* is closely related to *P. mutata* (Peck) Höhn. & Litsch.". The writer also examined a portion of the type of *P. kauffmanii* and agreed that it was indistinguishable from *P. heterocystidia*. Another specimen, BPI *81279*, in the University of Toronto Herbarium under *P. kauffmanii*, resembles *P. heterocystidia* closely except for an apparent lack of cystidia and should probably be referred to that species. In the present study, large masses of crystalline material were found in five specimens of *P. populnea* examined as well as in the specimens of *P. heterocystidia*. In both species the aggregates of crystals were never found associated with cystidium-like structures and it is therefore considered incorrect to refer to them as cystidia.

From the list of specimens of *P. heterocystidia* that were examined it may be seen that the description of the fungus has been based on collections from *Acer* spp., *Carpinus caroliniana*, *Fagus grandifolia*, *Juglans cinerea*, *Robinia pseudoacacia*, and unidentified hosts, from localities in Ontario, Quebec, Michigan, Pennsylvania, New York, and Kentucky. Burt (7) reports the species "On fallen limbs of gray birch, beech, maple, *Carpinus*, *Magnolia*, and other frondose species. Canada to Mississippi and westward to Missouri and

in Mexico. June to March. Common". It appears, therefore, that *P. heterocystidia* has a wide host range and geographic distribution. The lack of records of its occurrence on *Populus* may be significant.

Peniophora populnea (Peck) Burt, Ann. Missouri Botan. Garden, 13 : 324. 1926. (*non* Bourd. & Galz., Hymén. de France, p. 282. 1928).

Stereum populneum Peck, N. Y. State Museum Rept. 47 : 145. 1894.

Fructifications (Plate I, Figs. 5, 6) resupinate, effused, tough membranaceous, more or less adnate, even, cracking into small polygonal areas, drying pinkish drab and gray, 2.5YR4/3 to 5.0YR3/2, margin paler, narrow, very thin, radially byssoid, becoming more or less free. Section 175–355μ thick, distinctly yellowish-brown in color, the layer next the substratum consisting of longitudinally arranged hyphae, 2.0–3.6μ in diameter, bearing numerous clamp connections, walls thickened, contents hyaline and staining in phloxine or yellowish-brown, or lacking, the remaining portion consisting of ascending hyphae, cystidia, gloeocystidia, and basidia. Cystidia occurring throughout the hymenial layer but not protruding, 30.0–50.0 × 5.4–7.2μ, nodose-septate at the base, more or less cylindrical, with walls thick and refractive, the thickening usually greatest in a region 7.0–10.0μ back from the apex, incrusted in a tunicate manner, in potassium hydroxide solution the incrustation often splitting longitudinally and floating away from the cystidium (Plate II, Fig. 9); large compact masses of crystalline material present in subhymenial layers of five collections examined; gloeocystidia not abundant, 40.0–105.0 × 4.5–8.0μ, subtended by a clamp connection, cylindrical, flexuous, sometimes extending between the basidia but not protruding (Plate II, Fig. 10); basidia 30.0–40.0 × 6.0–7.0μ, clavate and occasionally swollen in the central region, with four slender, tapering sterigmata up to 6.0μ in length (Plate II, Fig. 8); basidiospores hyaline, cylindrical, slightly depressed on one side, apiculate, 10.8–13.5 × 3.6–4.0 (12.0 × 3.6)μ (Plate II, Fig. 11).

Specimens Examined:

Manitoba: on *Populus tremuloides*, Brereton Lake, Whiteshell Forest, Aug. 31, 1947, C. G. Riley, DAOM *21342*.

FIGS. 1 to 22. Structures present in fruit bodies. All ×720.

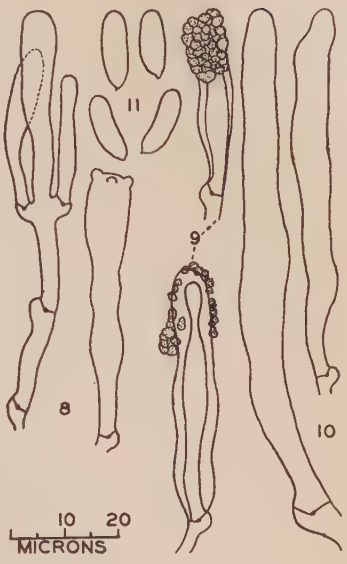
FIGS. 1 to 7. *Peniophora heterocystidia.* FIG. 1. Gloeocystidium from fruit body produced in nature. FIG. 2. Gloeocystidium from fruit body produced in culture. FIG. 3. Cystidia from fruit body produced in nature. FIG. 4. Cystidium from fruit body produced in culture. FIG. 5. Basidia from fruit body produced in nature. FIG. 6. Basidium from fruit body produced in culture. FIG. 7. Basidiospores from fruit body produced in nature.

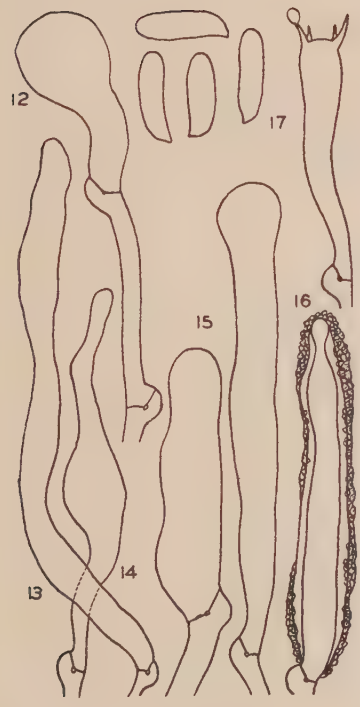
FIGS. 8 to 11. *Peniophora populnea.* All structures from fruit bodies produced in nature. Fig. 8. Stages in development of basidia. FIG. 9. Cystidia. FIG. 10. Gloeocystidia. FIG. 11. Basidiospores.

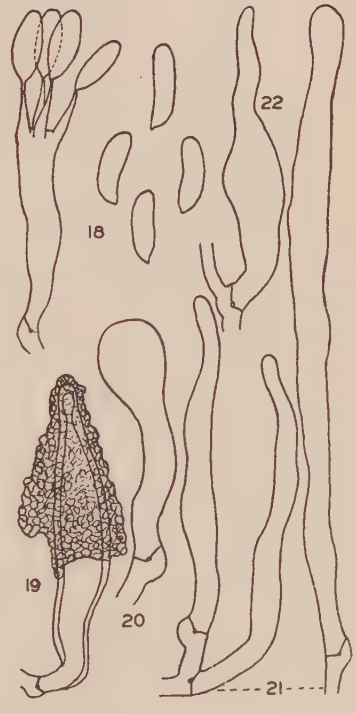
FIGS. 12 to 17. *Peniophora mutata.* All structures from collections DAOM *17214*, *17558*, and *17569*, on *Populus*. FIG. 12. Pyriform gloeocystidium. FIG. 13. Elongate, flexuous gloeocystidium. FIG. 14. Gloeocystidium with saccate base. FIG. 15. Cylindrical and clavate gloeocystidia. FIG. 16. Cystidium. FIG. 17. Basidium and basidiospores.

FIGS. 18 to 22. *Peniophora mutata.* All structures from collections DAOM *17215*, *17547*, and *17550* on hosts other than *Populus*. FIG. 18. Basidium and basidiospores. FIG. 19. Cystidium. FIG. 20. Pyriform gloeocystidium. FIG. 21. Elongate, flexuous gloeocystidia. FIG. 22. Gloeocystidium with saccate base.

PLATE II







Ontario: near Killarney Lodge, Algonquin Park, Sept. 28, 1938, H. S. Jackson, TRT *13611*; on *Populus*, Cartier Lake, Petawawa, July 13, 1935, J. E. Bier, DAOM *F8093* (TRT *13693*); north of Summit Golf Course, Richmond Hill, July 5, 1942, H. S. J., DAOM *10740* (TRT *17577*); Sept. 15, 1946, H. S. J., DAOM *17217* (TRT *21188*); Sept. 27, 1947, H. S. J., DAOM *17559* (TRT *22273*); Oct. 29, 1938, H. S. J., TRT *13621*; TRT *13649*; May 27, 1937, H. S. J., TRT *13723*.

Peniophora populnea was established by Peck (22) as *Stereum populneum* and transferred to *Peniophora* by Burt (8). The latter reports the species as lacking gloeocystidia and possessing heavily incrusted cystidia up to 20–25μ in diameter and paraphyses which he describes as "hair-like, colored, slender, 1–1½μ in diameter, branching at or near the tips into 2 or 3 short branches". In the present study gloeocystidia were observed, although they are not abundant, and could have been overlooked in the sections upon which Burt relied for his information, while the structures he called paraphyses may have been collapsed basidia. In mounts from fruit bodies of *P. populnea* brownish collapsed structures, including hyphae, cystidia, gloeocystidia, and basidia are much in evidence. The short sterigmata, which Burt describes, were probably in early stages of development, similar to those illustrated in Plate II, Fig. 8.

The fruit bodies on which the above description was based occurred on species of *Populus*, which is the only host genus recorded by Peck and Burt. It may be significant that *P. heterocystidia*, similar in many respects and probably closely related, has been recorded from many genera of broad-leaved trees but not from *Populus*, while *P. populnea* is known only from that host genus.

It should be noted that *P. populnea* Bourd. & Galz. and *P. populnea* (Peck) Burt are two distinct species, as is evident from a comparison of Bourdot and Galzin's (4) description and figure with the description in Burt's monograph and in the present paper.

Peniophora mutata (Peck) Höhn. & Litsch., K. Akad. Wiss. Wien Math.-Natw. Kl. Sitzber. 115, I: 1580. 1906.

Corticium mutatum Peck, N. Y. State Museum Rept. 43: 69. 1890.

Peniophora mutata Bres. in Bourd. & Galz., Soc. Myc. France Bul. 28 : 399. 1913; Burt, Ann. Missouri Botan. Garden, 12: 299. 1925.

Corticium allescheri Bres., Fungi Trident. 2 : 62. 1898.

Peniophora allescheri Sacc. & Syd., Syll. Fung. 16: 194. 1902; Burt, Ann. Missouri Botan. Garden, 12: 301. 1925.

Kneiffia allescheri Bres., Ann. Mycol. 1 : 100. 1903.

Gloeopeniophora allescheri Höhn. & Litsch., K. Akad. Wiss. Wien Math.-Natw. Kl. Sitzber. 117, I: 1082. 1908.

Peniophora cremea subsp. *allescheri* Bourd. & Galz., Hymén. de France, p. 304. 1928, *quantum ad typum, descr. excl.*

Peniophora cremea var. *allescheri* Litsch., Osterr. Bot. Zeitschr. 88 : 117. 1939, *quantum ad typum.*

TABLE I

COMPARISON OF STRUCTURES IN COLLECTIONS OF *Peniophora mutata*

	A			*B*		
	17214	17558	17569	17215	17547	17550
Gloeocystidia Types present	Pyriform numerous; elongate flexuous; elongate saccate	Pyriform very numerous	Stout cylindrical to clavate; few pyriform	Elongate saccate	Elongate flexuous	Pyriform numerous; elongate flexuous
Cystidia	Present in some areas, absent in others	Very rare	Numerous	Numerous	Numerous	Rare
Basidia Range of size in microns	40.5–54.0 × 7.2–9.0	45.0–63.0 × 7.2–9.0	37.0–45.0 × 6.3–7.2	31.0–54.0 × 7.2	40.5–63.0 × 6.3–7.2	31.0–40.0 × 6.0–8.0
Basidiospores Range of size in microns	11.7–14.4 × 3.6–4.5	11.7–16.2 × 3.6–4.5	11.7–15.3 × 3.6–4.5	9.9–13.5 × 3.6–4.5	10.8–13.5 × 3.6–4.0	9.9–13.5 × 3.0–4.0
Mean size in microns	13.0 × 4.0	13.4 × 3.8	13.5 × 3.8	11.6 × 3.9	12.7 × 3.6	12.7 × 3.6
Conidia Range of size in microns	11.7–17.1 × 3.0–4.0	12.6–18.9 × 3.0–4.0	12.6–18.0 × 3.0–4.0	11.7–17.1 × 2.7–4.0	9.9–13.5 × 2.7–3.6	10.8–15.3 × 2.7–3.6
Mean size in microns	15.0 × 3.9	15.3 × 3.4	14.8 × 3.6	13.9 × 3.3	11.4 × 2.8	12.5 × 3.0

Fructifications (Plate I, Figs. 7 to 9) resupinate, effused, membranaceous to fleshy, separable when moistened, very thick, uneven, occasionally tuberculate or with radial folds, sometimes cracking in drying, curling away from the fissures and revealing the white fibrillose subiculum, drying white to cream colored, 2.5Y8/4 to 10.0YR8/6, the margin white, radially byssoid. Section 350–1100μ thick, colorless, the hyphae 3.0–4.5(−6.3)μ in diameter, closely interwoven and parallel to the substratum in a narrow layer, then loosely arranged and ascending, walls becoming thickened and sometimes incrusted, the hymenial layer 60–100μ thick. Cystidia 30.0–75.0 × 7.0–11.0μ in the subhymenium, rare to numerous in different fruit bodies and in different parts of the same fruit body (Table I), nodose-septate at the base, irregularly cylindrical or tapering toward each end, walls thick and refractive, the thickening usually greatest 7.0–10.0μ back from the apex, heavily incrusted, with the incrustation extending down the hypha or concentrated in a tunicate structure which may split longitudinally and float away from the cystidium in mounts in aqueous potassium hydroxide (Plate II, Figs. 16, 19); gloeocystidia subtended by a clamp connection, of four general types which may be present in various combinations (Table I); (*a*) elongate, flexuous, 50.0–110.0 × 5.4–7.2μ (Plate II, Figs. 13, 21); (*b*) saccate toward the base, becoming elongate, 45.0–60.0 × 9.0–10.8μ (Plate II, Figs. 14, 22); (*c*) cylindrical to clavate, 45.0–70.0 × 9.0μ (Plate II, Fig. 15); (*d*) pyriform, 22.0–65.0 × 9.0–13.5μ (Plate II, Figs. 12, 20), all types in the subhymenium, type (*a*) extending between basidia and occasionally protruding up to 10.0μ; basidia 30.0–65.0 × 6.0–9.0μ, slender, clavate, with four sterigmata, broad at the base, tapering to a point, up to 9.0μ in length (Plate II, Figs. 17, 18); basidiospores 10.0–16.0 × 3.0–4.5 (12.8 × 3.8)μ, hyaline, cylindrical, inner side usually indented, apiculate (Plate II, Figs. 17, 18).

Specimens Examined:

British Columbia: on *Populus tremuloides*, Heffley Creek, Oct. 1, 1944, D. C. Buckland, DAOM *16002*; on *P. trichocarpa*, Quesnel, Sept. 14, 1948, W. G. Ziller, DAOM *21300*.

Manitoba: on *Populus*, Winnipeg, Sept. 16, 1932, I. Mounce and G. R. Bisby, DAOM *F5874*; July 6, 1927, G. R. B., DAOM *F6091*.

Ontario: on *Acer saccharum*, Dorset, Nov. 13, 1948, V. J. Nordin, DAOM *21309;* on *Acer*, Dorset, Aug., 1947, V. J. N., DAOM *17621*; Ottawa, Sept. 9, 1949, C. G. McKeen, DAOM *21624*; on *Populus*, Timagami, Aug. 21, 1930, L. O. Overholts, DAOM *F1893* (L. O. O. *13425*); New Durham, Brant Co., Oct. 29, 1934, R. F. Cain, DAOM *F5344* (TRT *6536*); south of Hatchley, Brant Co., Aug. 27, 1934, R. F. C., DAOM *F5356* (TRT *6514*); Huntsville, July 3, 1932, R. Macrae, DAOM *F6411*; north of Summit Golf Club, Richmond Hill, Sept. 15, 1946, H. S. Jackson, DAOM *17214* (TRT *21191*); Sept. 27, 1947, H. S. J., DAOM *17558* (TRT *22278*); Wilno, July 24, 1948, C. G. McKeen, DAOM *21205*; Papineau Twp., July 13, 1949, S. N. Linzon, DAOM *22316*; on *Tilia*, McKay Lake, Oct. 10, 1902, DAOM *F6357*; host not known, Ottawa, Oct. 3, 1883, J. Macoun, DAOM *F2778*.

Quebec: on *Acer*, Chelsea, Oct. 9, 1936, M. K. Nobles, DAOM *F7225*; I. Mounce and M. K. N., DAOM *F7661*; Hermit Lake, Gatineau Park, Sept. 25, 1947, M. K. N., DAOM 17547; Ridge Road, Gatineau Park, Sept. 4, 1949, C. G. McKeen, DAOM *21622*; on *Tilia americana*, Ridge Road, Gatineau Park, Sept. 4, 1949, C. G. M., DAOM *21621*; on *Tilia*, Farmer's Rapids, Sept. 18, 1931, I. M. and I. L. Conners, DAOM *F2132*; host not known, Ironsides, Sept. 18, 1891, J. Macoun, DAOM *F2780*; Beebe, July 30, 1931, I. M., DAOM *F6741*; Abbotsford, July 29, 1931, I. M., DAOM *F6742*; Chelsea, Oct. 9, 1936, I. M. and M. K. N., DAOM *F7248*; Nov. 6, 1936, I. M., DAOM *F7475*; Hermit Trail, Gatineau Park, Sept. 25, 1947, C. G. M., DAOM *17550*.

Prince Edward Island: on *Populus*, Brackley Point, Aug. 19, 1888, J. Macoun, DAOM *F2781*.

Locality not known: host not known, J. Macoun, DAOM *F2775*.

Michigan: on *Populus*, Lum, Oct. 12, 1947, H. S. Jackson, DAOM *17569*.

New York: on *Tilia*, King's Ravine, North of Frontenac Point, Cayuga Lake, Oct. 13, 1946, H. S. Jackson, DAOM *17215* (TRT *21346*).

Pennsylvania: on *Juglans cinerea*, Little Juniata River, Spruce Creek, Huntingdon Co., July 7, 1933, W. L. White, DAOM *F3807* (L. O. Overholts *16469*); on *Liriodendron tulipifera*, Charter Oak, Huntingdon Co., Mar. 17, 1934, L. O. O. and W. A. Campbell, DAOM *F3806* (L. O. O. *16668*); on *Populus grandidentata*, Pennsylvania State College, Nov. 12, 1932, L. L. Sluzalis, DAOM *F6540* (L. O. O. *15136*).

The first recorded collection of *Peniophora mutata* was made in 1890 in North America by Peck (21) who named it *Corticium mutatum*, the specific name being descriptive of the change from the folded and tuberculate nature of the hymenium in the moist condition to the thinner, rimose texture in the dried state. In 1906 von Höhnel and Litschauer (10) transferred the species to *Peniophora*. In 1913 Bourdot and Galzin (3), apparently unaware of von Höhnel and Litschauer's disposition of the species, also published its transfer to *Peniophora*, on Bresadola's authority. Burt (7) followed Bourdot and Galzin, referring to the species as *P. mutata* (Peck) Bres. in Bourdot and Galzin. Meanwhile a specimen collected in Bavaria by Allescher was made the basis for the species *Corticium allescheri* by Bresadola (5) in 1898. This was transferred to *Peniophora* by Saccardo and Sydow (25) in 1902, to *Kneiffia* by Bresadola (6) in 1903, and to *Gloeopeniophora* by von Höhnel & Litschauer (11), who had noted the gloeocystidia, in 1908. Rogers and Jackson (24), in discussing the synonomy of *Peniophora allescheri*, state, "Von Höhnel & Litschauer (1908), and Bourdot have both indicated that two species were included in the original material, the present one and *P. cremea* (Bres.) Sacc. & Syd. Bourdot's subspecies and Litschauer's variety are described from the '*cremea*' portion; nevertheless, being based on '*Allescheri*', and hence on its type, they must be included here."

Burt (7) appreciated the similarities between *P. mutata* and *P. allescheri* but recognized both species. He wrote "The type of *P. Allescheri* and

specimens of similar structure cited below differ so slightly from *P. mutata* that I have separated them from the latter only by all their gloeocystidia being of slender elongated form and perhaps specially differentiated middle portions of hyphae, while the gloeocystidia of *P. mutata* are terminal portions of hyphae and hyphal branches which are in many cases pyriform and in others afford indication by a pyriform base of having finally grown out from a pyriform body into an elongated gloeocystidium". Rogers and Jackson considered that the portion of the type of *P. allescheri* in the Burt herbarium "agrees extremely well, as Burt has pointed out, with the upper figure in Bresadola's plate; and equally well with Peck's *C. mutatum*", and concluded that *P. allescheri* (Bres.) Sacc. & Syd. should be placed in synonomy with *P. mutata* (Peck) Höhn. & Litsch. The present study confirms this conclusion.

As the list of specimens examined indicates, *Peniophora mutata* is widely distributed in Canada and the United States. Burt reported it from Canada, United States, the West Indies, Europe, and Japan. Its recorded host range is likewise extensive and includes species of *Populus* as well as many other genera of broad-leaved trees.

As stated previously the present study confirms the conclusion of Rogers and Jackson that *P. mutata* and *P. allescheri* should be combined. Gloeocystidia of four types were recognized in the specimens examined, the "elongate, flexuous" type probably being analogous to the "elongated, flexuous" gloeocystidia described for *P. allescheri*, and the "saccate toward the base, becoming elongate" and "pyriform" types being identical with the "pyriform" and pyriform becoming "elongated, flexuous" types described for *P. mutata*, by Burt. While Bourdot and Galzin do not distinguish between cystidia and gloeocystidia in *P. mutata*, it seems probable that the first two types of "cystidia", which they describe as "les unes à parois minces, fusoïdes ou cylindriques, 40–115 × 4–9μ; quelques-unes capitées vésiculaires, 60–70 × 14–18μ", agree with the elongate and pyriform gloeocystidia described by Burt and in the present paper. In the 12 collections of *P. mutata* listed in Table III, proved to belong to the same species by interfertility tests as will be shown in a later section, the four types of gloeocystidia occurred in various combinations, frequently with as many as three types in a single fruit body. As an illustration, the combinations of types of gloeocystidia found in six fruit bodies are recorded in Table I. Hence the separation of *P. mutata* and *P. allescheri* on the basis of the types of gloeocystidia is not justified.

Irregularities in the distribution of the cystidia were noted by Burt, who wrote, "sometimes so few present as to be found only after examination of several sections", by Bourdot and Galzin who found them "très inégalement distribuées", and in the present study. As shown in Table I they varied from numerous to rare or apparently lacking in some areas. It is of interest that the fruit body of *P. mutata*, DAOM *17550*, in which cystidia were of rare occurrence, was growing on the same log and confluent with the fruit body of *P. heterocystidia*, DAOM *17551*, in which numerous cystidia and large aggre

gates of crystals were found. *P. mutata* and *P. heterocystidia* were found growing together in three other collections in the Mycological Herbarium, Department of Agriculture, Ottawa, *F3807* under *P. mutata*, *P. mutata F7225* and *P. heterocystidia F7233*, *P. mutata 21309* and *P. heterocystidia 21308*, shown in Plate I, Fig. 7.

Previous writers have recorded a considerable variation in spore size, Burt giving the range as 8–16 × 3–4μ, Bourdot and Galzin as 8–16 × 3–5μ. In the present study the range was found to be 10.0–16.0 × 3.0–4.5μ, which is in close agreement, with a mean of 12.8 × 3.8μ. As shown in Table I the range and mean were higher in collections on *Populus* than in collections from other hosts.

It is evident from the descriptions of the fruit bodies that *P. heterocystidia*, *P. populnea*, and *P. mutata* are so similar as to indicate close relationship. They are alike in their structure, which consists of a basal layer of hyphae parallel to the substratum and an ascending subhymenial layer made up of nodose-septate hyphae, in the presence of cystidia and gloeocystidia, and in the shape and size of basidiospores. Differences are to be sought not in the presence or absence of structures or in distinct differences in their sizes (Table II) but in their relative numbers or in slight difference in shape. Color, thickness, and host range offer additional diagnostic criteria.

TABLE II

COMPARISON OF SIZES OF STRUCTURES IN *Peniophora heterocystidia*, *P. populnea*, AND *P. mutata*

	Peniophora heterocystidia	*Peniophora populnea*	*Peniophora mutata*
Cystidia Range of size in microns	22.0–55.0 × 5.0–9.0	30.0–50.0 × 5.4–7.2	30.0–75.0 × 7.0–11.0
Gloeocystidia Range of size in microns	40.0–100.0 × 6.0–10.0	40.0–105.0 × 4.5–8.0	22.0–110.0 × 5.4–13.5
Basidia Range of size in microns	15.0–30.0 × 4.5–6.3	30.0–40.0 × 6.0–7.0	30.0–65.0 × 6.0–9.0
Basidiospores Range of size in microns	11.0–15.0 × 3.5–4.5	10.8–13.5 × 3.6–4.0	10.0–16.0 × 3.0–4.5
Mean size in microns	12.2 × 3.8	12.6 × 3.6	12.8 × 3.8
Conidiophores Diameter in microns	5.4–9.9	4.5–9.9	8.0–15.3
Conidia Range of size in microns	9.0–15.0 × 2.7–3.6	11.7–17.1 × 3.6–4.0	10.0–18.9 × 2.7–4.5
Mean size in microns	12.0 × 3.3	14.3 × 3.7	13.8 × 3.3

Thus *P. heterocystidia* is usually pinkish drab to cinnamon drab, 200–400μ thick, and is found on broad-leaved trees belonging to genera other than *Populus*. In section it is colorless or with color restricted to the hymenium, its basidia and cystidia are swollen toward the base, its gloeocystidia are numerous and irregularly cylindrical and flexuous, and large masses of crystalline material are usually present. *P. populnea* is similar in color and thickness but is restricted to species of *Populus*. In section it is noteworthy for its yellow or brown color, the result of a preponderance of structures which have become collapsed and yellowish-brown or empty. The gloeocystidia are not numerous and are usually more or less cylindrical rather than obclavate as in *P. heterocystidia*. As in the latter species, large masses of crystalline material may occur in the subhymenium. The fruit bodies of *P. mutata* are paler than those of the other species, with a cream or yellowish tinge, are usually much thicker, up to 1100μ, and may be found on a wide range of broad-leaved trees. In section it is colorless and is notable for the variety in shapes of gloeocystidia.

TABLE III

LIST OF FRUIT BODIES FROM WHICH CULTURES WERE ISOLATED

Fungus	Collection No.		Host	Locality
	DAOM	TRT		
Peniophora heterocystidia	*F7233*		*Acer* sp.	Chelsea, Que.
	10899	*17578*	*Carpinus caroliniana*	Toronto, Ont.
	17216	*21272*	*Carpinus* sp.	Richmond Hill, Ont.
	17551		?	Gatineau Park, Que.
	17568		*Carpinus* sp.	La Peer, Mich.
	17587		*Acer* sp.	Gatineau Park, Que.
	21301		*Fagus grandifolia*	Bolton, Peel Co., Ont.
	21308		*Acer saccharum*	Dorset, Ont.
Peniophora populnea	*10740*		*Populus* sp.	Richmond Hill, Ont.
	17217	*21188*	*Populus* sp.	Richmond Hill, Ont.
	17559		*Populus* sp.	Richmond Hill, Ont.
Peniophora mutata	*F7991**	*6535*	*Tilia americana*	Hatchley, Ont.
	*17214**	*21191*	*Populus* sp.	Richmond Hill, Ont.
	*17215**	*21346*	*Tilia americana*	Cayuga Lake, N.Y.
	*17547**		*Acer* sp.	Gatineau Park, Que.
	*17550**		?	Gatineau Park, Que.
	17558		*Populus* sp.	Richmond Hill, Ont.
	17569		*Populus* sp.	Lum, Mich.
	21205		*Populus* sp.	Wilno, Ont.
	21300		*Populus trichocarpa*	Quesnel, B.C.
	21621		*Tilia americana*	Gatineau Park, Que.
	21622		*Acer* sp.	Gatineau Park, Que.
	21624		*Acer* sp.	Ottawa, Ont.
	22316		*Populus* sp.	Papineau Twp., Ont.

* *Originally identified as Peniophora allescheri.*

Cultural Studies

The descriptions of cultures have been based on cultures in the collection at the Division of Botany and Plant Pathology, Science Service, Ottawa, isolated from specimens deposited in the Mycological Herbarium, Department of Agriculture, Ottawa. The collections from which cultures were obtained are listed in Table III. Cultural characters were determined according to the method used by Nobles (20) and descriptions have been prepared in conformity with that publication.

Peniophora heterocystidia

Polysporous Cultures

KEY PATTERN: 1 (1, 2) 1 1 1 2 1 2 2 1 (2, 3)

CULTURAL CHARACTERS: (Plate III, Figs. 1 to 3)

GROWTH CHARACTERS.—Growth moderately rapid, plates covered in three (−four) weeks (from inoculum at edge). Advancing zone even, hyaline, appressed. Mat white at first, showing tinges of pinkish-buff, 7.5YR8/5 in older parts after two to three weeks to 10.0YR8/5 in irregular areas over most of mat in some isolates after six weeks, at first raised, fine cottony, extending up side of Petri dish, after two to three weeks becoming collapsed in older parts and forming irregular subfelty areas or minute cottony balls, with a suggestion of zonation and sectoring in some isolates, usually forming fruit bodies after five weeks, those against side wall of Petri dish usually irregular, fine spinelike, those on surface similar or skinlike, resupinate, more or less circular, from 3–4 mm. to 2 cm. in diameter, 7.5YR8/5 in color. Reverse unchanged in most isolates, partially bleached after five weeks in two isolates. No odor. On gallic and tannic acid agars diffusion zones moderately strong, no growth on gallic acid agar, diameter trace to 1.3 cm. on tannic acid agar.

HYPHAL CHARACTERS.—*Advancing zone:* hyphae hyaline, thin-walled, contents staining in phloxine, nodose-septate, branching at and occasionally between the septa, 1.5–4.5μ diameter. *Aerial mycelium:* (*a*) hyphae as in advancing zone, more frequently branched; (*b*) conidiophores (Plate IV, Fig. 2) appearing within a week, abundant in all stages of formation throughout the six weeks during which the cultures were observed, arising as irregular terminal or lateral swellings 5.4–9.9μ in diameter, bearing conidia on slender, tapering sterigmata up to 2.0μ in length, collapsing when conidia are mature; (*c*) conidia (Plate IV, Fig. 3) 9.0–15.0 × 2.7–3.6 (12.0 × 3.3)μ, cylindrical, slightly curved, inconspicuously apiculate; (*d*) hyphae in colored areas empty or with yellowish-brown contents, agglutinated in more or less parallel strands. *Fruit body:* (*a*) colored hyphae as in aerial mycelium; (*b*) hyaline hyphae, frequently nodose-septate, with numerous branches, closely interwoven; (*c*) obclavate cystidia, (*d*) gloeocystidia, (*e*) basidia, and (*f*) basidiospores as described for fruit bodies above (Plate II, Figs. 2, 4, 6).

Monosporous Cultures

These (Plate III, Figs. 4 to 6) are similar in appearance to polysporous cultures but generally produce less aerial mycelium and more numerous conidiophores (Plate IV, Fig. 1). Like the polysporous cultures they produce fruit bodies with cystidia, gloeocystidia, basidia, and basidiospores similar in all respects to those produced in polysporous cultures except that they arise from simple-septate rather than nodose-septate hyphae.

Peniophora populnea

Polysporous Cultures

KEY PATTERN: 1 (1, 2) 1 1 9 2 1 2 2 2 (2, 3)

CULTURAL CHARACTERS: (Plate III, Figs. 7 to 9)

GROWTH CHARACTERS.—Growth moderately rapid, plates covered in three weeks. Advancing zone even, hyaline, appressed. Mat white, developing tinges of cream to pinkish-buff, 2.5Y8/5 to 7.5YR7/5, after four weeks where mycelium is grown against side wall, at first somewhat raised in narrow zone, fine cottony, soon becoming appressed, firm cottony to subfelty, extending up side wall of Petri dish. Reverse unchanged or bleached after six weeks. Odor none. On gallic and tannic acid agars diffusion zones moderately strong to strong, no growth on gallic acid agar, diameter trace to 1.7 cm. on tannic acid agar.

HYPHAL CHARACTERS.—*Advancing zone:* hyphae hyaline, thin-walled, contents staining in phloxine, nodose-septate, branching at and occasionally between septa, 1.5–4.5μ in diameter. *Aerial mycelium:* (*a*) hyphae as in advancing zone, frequently with numerous irregular branches; (*b*) conidiophores (Plate IV, Figs. 4, 5) appearing within a week, not abundant, arising as terminal or lateral branches on hyphae, the tip enlarging to form a pyriform head, the distal portion of which is covered with slender, tapering sterigmata up to 2.0μ in length, becoming collapsed when conidia are mature; (*c*) conidia (Plate IV, Fig. 6) cylindrical to slightly curved, somewhat apiculate, 11.7–17.1 × 3.6–4.0 (14.3 × 3.7)μ.

Monosporous Cultures

These are similar in appearance to the polysporous cultures but exhibit a wide variation in growth rate. Microscopically they differ only in the possession of simple-septate rather than nodose-septate hyphae (Plate IV, Fig. 7).

Peniophora mutata

Polysporous Cultures

KEY PATTERN: 1 (1, 2) 1 1 9 2 1 2 2 2 3

CULTURAL CHARACTERS: (Plate III, Figs. 10 to 15)

GROWTH CHARACTERS.—Growth moderately rapid, plates covered in three (−four) weeks. Advancing zone even, hyaline, appressed. Mat white except for tinge of dull brown, 10.0YR4/3–8/3, over roll of mycelium against side wall after four to six weeks, at first raised, fine cottony-woolly, becoming collapsed and subfelty in older part after four weeks but remaining cottony at outer limits and in roll against side wall. Reverse bleached after three (−six) weeks. No odor. On gallic and tannic acid agars diffusion zones moderately strong, no growth on gallic acid agar, no growth or a trace on tannic acid agar.

HYPHAL CHARACTERS.—*Advancing zone:* hyphae hyaline, thin-walled, contents staining in phloxine, nodose-septate, branching at and occasionally between the septa, 1.5–4.5μ in diameter. *Aerial mycelium:* (*a*) hyphae as in advancing zone, with numerous clamp connections and many irregular branches; (*b*) conidiophores (Plate IV, Figs. 8, 12) appearing within a week, very numerous in some isolates, arising as terminal or lateral branches in the hyphae, becoming pyriform at the apex, 8.0–15.3μ in diameter, the distal portions covered with slender, tapering sterigmata up to 2.0μ in length, collapsing when conidia are mature; (*c*) conidia (Plate IV, Figs. 10, 11) hyaline, cylindrical or slightly curved, inconspicuously apiculate, 10.0–18.9 × 2.7–4.5 (13.8 × 3.3)μ.

The description of conidiophores and conidia agrees with that given by Nobles (18) for cultures of *P. allescheri*. The conidia, like the basidiospores, vary in size in the different collections, those from fruit bodies on *Populus* spp. being somewhat larger than those isolated from fruit bodies on hosts other than *Populus* spp.

FIGS. 1 to 15. Cultures grown on malt extract agar in the dark, those in row at left two weeks old, in center row four weeks old, in row at right six weeks old.

FIGS. 1 to 6. *Peniophora heterocystidia*, DAOM *17216*. FIGS. 1 to 3. Polysporous culture. FIGS. 4 to 6. Monosporous culture.

FIGS. 7 to 9. *Peniophora populnea*, DAOM *17217*, polysporous culture.

FIGS. 10 to 15. *Peniophora mutata*. FIGS. 10 to 12. DAOM *17558* from *Populus*, polysporous culture. FIGS. 13 to 15. DAOM *17547* from *Acer*, polysporous culture.

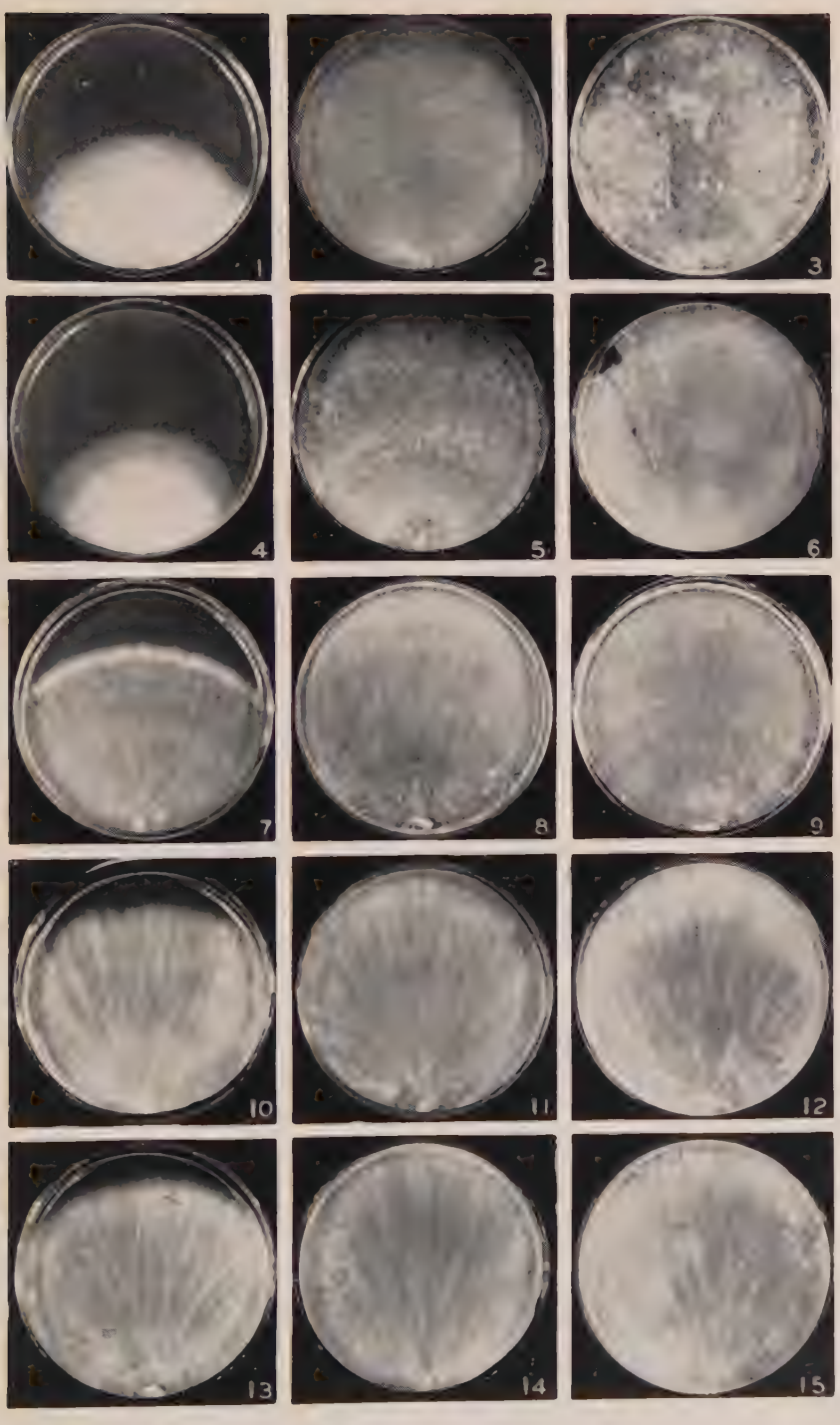

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

PLATE IV

FIGS. 1 to 12. Structures present in cultures. All ×720.

FIGS. 1 to 3. *Peniophora heterocystidia.* FIG. 1. Conidiophores produced in monosporous culture. FIG. 2. Conidiophores produced in polysporous cultures. FIG. 3. Conidia.

FIGS. 4 to 7. *Peniophora populnea.* FIGS. 4, 5. Conidiophores produced in polysporous cultures. FIG. 6. Conidia. FIG. 7. Conidiophores produced in monosporous culture.

FIGS. 8 to 12. *Peniophora mutata.* FIGS. 8 to 10. Structures from cultures isolated from fruit bodies DAOM *17214*, *17558*, and *17569* on *Populus* spp. FIG. 8. Conidiophores produced in polysporous cultures. FIG. 9. Conidiophores produced in monosporous culture. FIG. 10. Conidia. FIGS. 11 and 12. Structures from cultures isolated from fruit bodies DAOM *17215*, *17547*, and *17550* on hosts other than *Populus*. FIG. 11. Conidia. FIG. 12. Conidiophores produced in polysporous cultures.

Monosporous Cultures

These are similar in appearance to polysporous cultures but generally produce relatively less aerial mycelium and more numerous conidiophores (Plate IV, Fig. 9) and grow more slowly. The hyphae are simple-septate.

The cultures of *P. heterocystidia*, *P. populnea*, and *P. mutata* are, like the fruit bodies, so similar as to indicate close relationship. Both polysporous and monosporous isolates of *P. heterocystidia* produce typical fruit bodies in culture, which makes possible their separation from cultures of the other two species which do not fruit under the conditions provided. *P. populnea* and *P. mutata* are separable only on the basis of diameter of conidiophores, which appears to be significantly larger in *P. mutata*.

In all three species, the germination of a single conidium, borne on either a polysporous or monosporous culture, produced a mycelium with simple septa, similar in all characters to a culture resulting from the germination of a single basidiospore.

Occurrence of Conidia in Nature

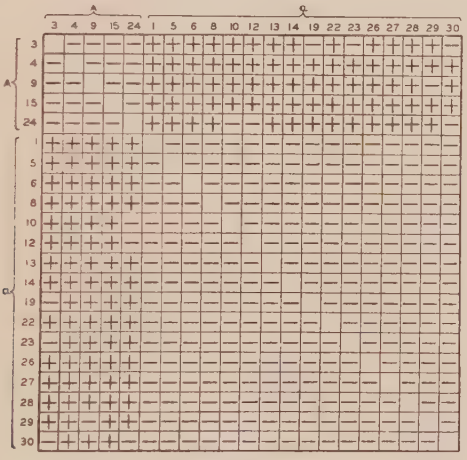
Conidiophores are produced with such regularity in cultures of *Peniophora heterocystidia*, *P. populnea*, and *P. mutata* that it was expected that they might be of some significance in the life cycle of these fungi in nature. Consequently a search was made for them, especially in very young portions of the fruit bodies. In October, 1948, Prof. H. S. Jackson sent the writer a piece of bark of *Fagus* bearing small areas of *P. heterocystidia* (DAOM *21301*) in which conidiophores appeared as well as basidia (Plate I, Fig. 4). No other conidiophores were found although a large number of young collections were examined. Later the attention of the author was directed to a paper by Rishbeth (23) on the biology of *Fomes annosus* Fr., in which he stated "When it was required merely to demonstrate the presence or absence of *F. annosus* in wood samples these were cleaned, wrapped in newspaper, thoroughly wetted with tap-water, and kept in glass containers at room temperature (17°–20° C.). Under such conditions the presence of *F. annosus* was indicated by the production of conidia, usually after 4–5 days . . . . These conidia are borne as characteristic heads, as originally described by Brefeld (1889), and are easily recognizable under the low power of the microscope". A collection of *Peniophora heterocystidia* (DAOM *22547*) made on October 10, 1950, was placed in a moist chamber. It was examined at intervals for conidiophores and these were found in abundance on October 16, even in the older parts of the fruit bodies. A photograph of the specimen is reproduced in Plate I, Fig. 3. It would appear that the production of conidia in *P. heterocystidia* depends on proper moisture conditions. Moisture could also play a part in the dissemination of the conidia, since they are easily removed from the conidiophores by touching them with a loopful of water.

Although it has been shown that, in artificial culture, the conidium generation can repeat the basidiospore generation it is doubtful if this occurs to any extent in nature, since in the few cases where conidiophores were observed

they were produced along with basidia. It appears that conidia are not produced with enough regularity to be of importance in the dissemination of the fungus in nature. However, there is the possibility that conidiophores and conidia may be produced before any conspicuous fruit body appears.

Interfertility Studies

As reported in the section on cultural characters, polysporous cultures of *Peniophora heterocystidia*, *P. populnea*, and *P. mutata* were found to have hyphae with clamp connections, while monosporous cultures had hyphae with simple septa. Hence, it was assumed that the three species are heterothallic. To substantiate this, 21 monosporous mycelia from isolate DAOM *17216* of *P. heterocystidia* were paired in all possible combinations, and the resulting mycelia examined for the presence of clamp connections. The results are shown in Text-fig. 1, where a (+) indicates the presence of clamp connections and a (−) their absence. The mycelia fall into two groups, a member of one

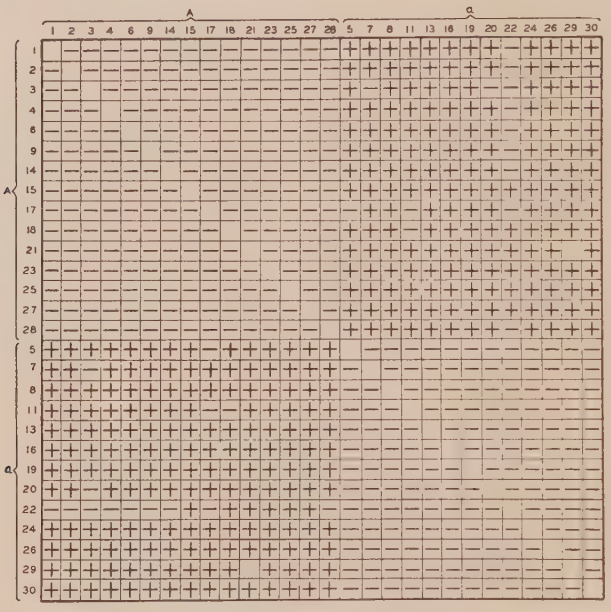


TEXT-FIG. 1. Results obtained by pairing in all possible combinations, 21 monosporous mycelia from *Peniophora heterocystidia*, fruit body DAOM *17216*.

group, designated by the symbol *A*, producing clamp connections when paired with any member of the other group, *a*, but not with a member of its own group. The isolate is, therefore, heterothallic, that is, hermaphroditic, self-sterile, and interfertile, and of the bipolar type of interfertility.

Similarly, 28 monosporous mycelia from isolate DAOM *17559* of *P. populnea* were paired in all possible combinations and the resulting mycelia examined for the presence of clamp connections. The results, presented in Text-fig. 2, show that the mycelia fall into two groups and that the isolate exhibits the bipolar type of interfertility. Pairings between 30 monosporous mycelia from isolate DAOM *17558* of *P. mutata*, seven monosporous mycelia of isolate DAOM *17214*, and seven of isolate DAOM *17215*, of which the results are

shown in Text-figs. 3, 4, and 5 demonstrated that in each case the mycelia fall into two groups. These results agree with those obtained by Nobles (18) who showed that *P. allescheri* exhibits the bipolar type of interfertility.

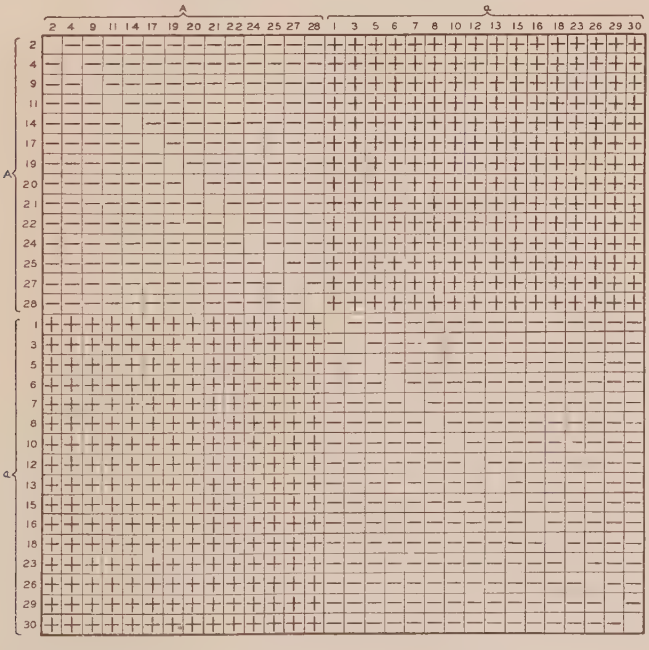


TEXT-FIG. 2. Results obtained by pairing in all possible combinations, 28 monosporous mycelia from *Peniophora populnea*, fruit body DAOM *17559*.

Various students of heterothallic Hymenomycetes have argued that, if monosporous mycelia from two different fruit bodies are interfertile, the fruit bodies belong to the same species. Conversely, the failure of monosporous mycelia from different bodies to pair in such a way as to form hyphae with clamp connections indicates that the fruit bodies belong to different species. However, groups of incompatible isolates within a species have been reported by certain workers. Thus, Vandendries (26, 27), in his studies on American and European forms of *Coprinus micaceus* Fr., found that fertility was the rule between isolates from the same region, sterility was the rule between isolates from widely separated European regions, fertility was the rule between American isolates although there were many exceptions in isolates from widely separated regions, and, finally, sterility was general between American and European isolates. Barnett (1), working with seven collections of *Auricularia auricula-judae* (Fr.) Schröt., found complete compatibility between five of the isolates while the other two were partially or completely incompatible with members of the first group. These two were growing on coniferous hosts and had longer spores, on the average, than the first five, which were on broad-leaved trees. The five of the first group were collected in Nebraska, North Carolina, and Iowa, the two on coniferous hosts in New York and Colorado.

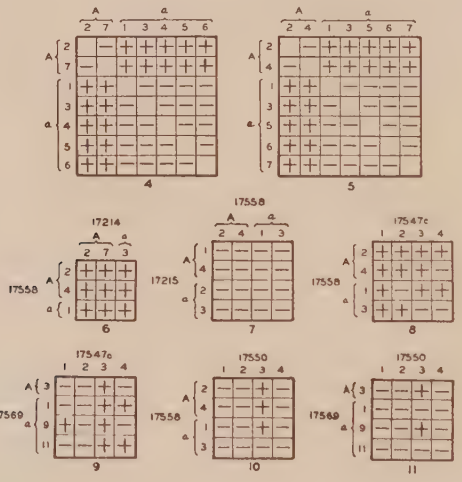
Mounce and Macrae (16) studied 52 isolates of *Fomes pinicola* (Sw.) Cooke of North American, European, and Japanese origin, and found that the cultures could be divided into three groups, a large Group *A* and a small Group *B* of North American origin, and a Group *C* of non-American origin. The isolates of each of these groups are compatible among themselves, those of Group *A* are incompatible with members of Group *B*, while those of Group *C* are "almost completely compatible with Group *A*, and *only partially incompatible with Group B.*" They state that "Since members of Group *A* have been found on each host species on which a member of Group *B* occurred, and in each locality, it seems definite that incompatibility in *Fomes pinicola* is not influenced primarily either by host or geographical distribution".

A parallel to the situation in *Fomes pinicola* is reported by Macrae (14) in *Polyporus abietinus* (Dicks.) Fr. This latter species has morphologically different forms of the hymenial surface, which are classed as poroid, lamellate, and irpicoid. As in *Fomes pinicola*, the North American poroid forms fall into two groups on the basis of their pairing reactions, the members of each group being compatible among themselves, intersterile, but partially compatible with poroid forms of non-American origin. The lamellate forms are compatible among themselves, incompatible with both American poroid groups, and partially incompatible with the European poroid groups. Macrae suggests that "In the lamellate collections inability to pair with the poroid form has been accompanied or followed by morphological changes and a new form or variety has arisen".



TEXT-FIG. 3. Results obtained by pairing in all possible combinations, 30 monosporous mycelia from *Peniophora mutata*, fruit body DAOM *17558*.

The striking similarities in the morphology of the fruit bodies, in the cultural characters, and in the type of interfertility, of *Peniophora heterocystidia*, *P. populnea*, and *P. mutata* suggested that an analysis of their reactions when paired might provide valuable information about their relationships. Monosporous mycelia were isolated from eight collections determined as *P. heterocystidia*, three determined as *P. populnea*, and 13 determined as *P. mutata* (or *P. allescheri*) on the basis of the morphological characters of the fruit bodies. These are listed in Table III. In most of the tests four monosporous mycelia from one collection were paired with four from another, and the resulting mycelia examined for the presence of clamp connections, denoted by (+) in Text-figs. 6 to 12, indicating fertility or compatibility, or their absence, denoted by (−), indicating sterility or incompatibility. The tables shown in Text-figs. 6 to 12 are representative of the results obtained from 137 such pairings between collections, Text-fig. 6 illustrating complete fertility, Text-fig. 7 complete sterility, Text-figs. 8 to 12 partial fertility or compatability. The results are summarized in Text-fig. 13, where (+) indicates complete fertility between collections, as in Text-fig. 6, (−) indicates complete sterility, as in Text-fig. 7, (±) partial fertility in which half or more of the combinations



TEXT-FIGS. 4–11.

TEXT-FIG. 4. Results obtained by pairing in all possible combinations, seven monosporous mycelia from *Peniophora mutata*, fruit body DAOM *17214*.

TEXT-FIG. 5. Results obtained by pairing in all possible combinations, seven monosporous mycelia from *Peniophora mutata*, fruit body DAOM *17215*.

TEXT-FIG. 6. Results obtained by pairing three monosporous mycelia from *Peniophora mutata*, DAOM *17214*, with three monosporous mycelia from *P. mutata*, DAOM *17558*.

TEXT-FIG. 7. Results obtained by pairing four monosporous mycelia from *Peniophora mutata*, DAOM *17215*, with four monosporous mycelia from *P. mutata*, DAOM *17558*.

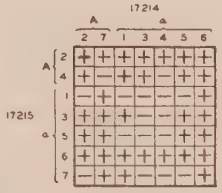
TEXT-FIG. 8. Results obtained by pairing four monosporous mycelia from *Peniophora mutata*, DAOM *17547c* with four monosporous mycelia from *P. mutata*, DAOM *17558*.

TEXT-FIG. 9. Results obtained by pairing four monosporous mycelia from *Peniophora mutata*, DAOM *17547c*, with four monosporous mycelia from *P. mutata*, DAOM *17569*.

TEXT-FIG. 10. Results obtained by pairing four monosporous mycelia from *Peniophora mutata*, DAOM *17558*, with four monosporous mycelia from *P. mutata*, DAOM *17550*.

TEXT-FIG. 11. Results obtained by pairing four monosporous mycelia from *Peniophora mutata*, DAOM *17569*, with seven monosporous mycelia from *P. mutata*, DAOM *17550*.

were fertile, as in Text-figs. 8 and 12, (∓) partial fertility in which less than half of the combinations were fertile, as in Text-figs. 9, 10, and 11. In pairings between mycelia from the same fruit body the sign (±) symbolizes the bipolar type of interfertility.



TEXT-FIG. 12. Results obtained by pairing seven monosporous mycelia from *Peniophora mutata*, DAOM 17214, with seven monosporous mycelia from *P. mutata*, DAOM *17215*.

From the summary in Text-fig. 13 it can be seen that, on the basis of the reactions of their monosporous mycelia in interfertility tests, the fruit bodies fall into three groups that coincide with those established on the basis of the morphological characters of the fruit bodies. The isolates from specimens identified as *P. heterocystidia* are compatible among themselves and incompatible with isolates from each of the other species. Similarly, the isolates from specimens identified as *P. populnea* are compatible among themselves and incompatible with isolates from *P. heterocystidia* and *P. mutata*. The isolates from specimens which had been identified as *P. mutata*, including

TEXT-FIG. 13. Results obtained by pairing, in all possible combinations, series of monosporous mycelia from different fruit bodies of *Peniophora heterocystidia*, *P. populnea*, and *P. mutata*.

those originally assigned to *P. allescheri*, are incompatible with isolates from the other species. Thus, interfertility tests have corroborated the separation based on the morphological characters of the fruit bodies and have confirmed the validity of the three species.

It is to be noted that cultures from fruit bodies determined as *P. mutata* fall into two groups, designated in Text-fig. 13 by *A* and *B*. Members of each group are completely compatible with other members of the same group but when members of Group *A* are paired with those of Group *B* partial compatibility usually is found, although in one cross, 21622 × 17214, complete fertility and in two, 17215 × 17558 and 17215 × 17569, complete sterility occurred. From the data in Table III it is obvious that the groups do not coincide with the original groups under *P. allescheri* and *P. mutata* since isolates from fruit bodies assigned to *P. allescheri* fall in both groups. This supports the evidence obtained from the examination of fruit bodies and cultures that *P. allescheri* should be combined with *P. mutata*. There appears to be no correlation between the groups and geographical distribution although the data are insufficient to be conclusive. Members of Group *A* were collected in localities in British Columbia, Ontario, and Michigan, those of Group *B* in Ontario, Quebec, and New York. To date, fruit bodies of the two Groups have not been found in the same vicinity. Correlation exists, however, between the Groups and the hosts on which the fruit bodies were growing, all the members of Group *A* having been collected on species of *Populus*, all members of Group *B* on broad-leaved hosts other than *Populus*. It would appear, therefore, that in *P. mutata*, compatibility is associated with host.

In a critical examination of three fruit bodies and their cultures from *Populus*, belonging to Group *A*, and three from hosts other than *Populus*, belonging to Group *B* (Table I), it was found that fruit bodies and cultures are indistinguishable except in sizes of basidiospores and conidia, for which the range and mean are slightly higher in those from *Populus*. The differences are not considered sufficient to warrant the use of a varietal name.

Certain species and varieties of other fungi are restricted to *Populus*. For example, Lloyd (13) in his description of *Radulum casearium* (Morgan) Lloyd, states that "I found it in quantity at Timagami, in northern Ontario, growing for wide extent, on poplar". All the specimens of the species in the Mycological Herbarium, Department of Agriculture, Ottawa, are on this host genus. *Fomes nigricans* var. *populinus* was segregated by Neuman on the basis of morphological differences, and cultural differences were recognized by Campbell (9) who transferred it to *Fomes igniarius* var. *populinus* (Neuman) Campbell. *Peniophora populnea* was described by Burt from *Populus tremuloides* and all of the collections in the University of Toronto and the Ottawa herbaria are on *Populus*.

It is interesting to compare *P. heterocystidia* and *P. populnea* with *P. mutata*, Groups *A* and *B*. *P. populnea* and *P. mutata* Group *A* are found only on species of *Populus*, while *P. heterocystidia* and *P. mutata* Group *B* are on various broad-leaved trees other than *Populus*. *P. mutata* Groups *A* and *B*

are for all practical purposes indistinguishable in cultures and in fruit bodies while *P. heterocystidia* and *P. populnea*, although similar, can be separated. Monosporous mycelia of *P. mutata* Groups *A* and *B* although occasionally completely incompatible are usually partially compatible. It is suggested that as a result of the partial inability of *P. mutata* Groups *A* and *B* to pair and their occurrence on different hosts, morphological changes may take place which will give rise to separate species. It seems possible that *P. heterocystidia* and *P. populnea* have followed a similar line of development.

References

1. Barnett, H. L. Studies in the sexuality of the Heterobasidiae. Mycologia, 29 : 626–649. 1937.
2. Biggs, R. The species concept in *Corticium coronilla*. Mycologia, 29 : 686–706. 1937.
3. Bourdot, H. and Galzin, A. Hyménomycètes de France. IV. Corticiés. Soc. Mycol. France Bull. 28 : 350–409. 1913.
4. Bourdot, H. and Galzin, A. Hyménomycètes de France. Paris, 1928.
5. Bresadola, J. Fungi Tridentini, 2 : 62. 1898.
6. Bresadola, J. Fungi polonici. Ann. Mycol. 1 : 7–131. 1903.
7. Burt, E. A. The Thelephoraceae of North America. XIV. *Peniophora*. Ann. Missouri Botan. Garden, 12 : 213–357. 1925.
8. Burt, E. A. The Thelephoraceae of North America. XV. *Corticium*. Ann. Missouri Botan. Garden, 13 : 173–354. 1926.
9. Campbell, W. A. The cultural characteristics of the species of *Fomes*. Bull. Torrey Botan. Club, 65 : 31–69. 1938.
10. Höhnel, F. v. and Litschauer, V. Beiträge zur Kenntnis der Corticieen. K. Akad. Wiss. Wien Math.-Natw. Klasse Sitzber. 115 (Abt. I) : 1549–1620. 1906.
11. Höhnel, F. v. and Litschauer, V. Beiträge zur Kenntnis der Corticieen (III. Mitteilung). K. Akad. Wiss. Wien Math.-Natw. Klasse Sitzber. 117 (Abt. I) : 1081–1124. 1908.
12. Lanjouw, J. and Stafleu, F. A. Index Herbariorum. Part 1. The herbaria of the world. Utrecht. 1952.
13. Lloyd, C. G. The genus *Radulum*. *In* Mycological writings. 5. 1917.
14. Macrae, R. Genetical and sexuality studies in some higher Basidiomycetes. Thesis. University of Toronto. 1941.
15. Mounce, I. and Macrae, R. The behavior of paired monosporous mycelia of *Fomes roseus* (Alb. & Schw.) Cooke and *Fomes subroseus* (Weir) Overh. Can. J. Research, C, 15 : 154–161. 1937.
16. Mounce, I. and Macrae, R. Interfertility phenomena in *Fomes pinicola*. Can. J. Research, C, 16 : 354–376. 1938.
17. Munsell Color Company. Munsell book of color. Munsell Color Company, Inc., Baltimore, Maryland. 1929–1942.
18. Nobles, M. K. Conidial formation, mutation and hybridization in *Peniophora allescheri*. Mycologia, 27 : 286–301. 1935.
19. Nobles, M. K. A contribution toward a clarification of the *Trametes serialis* complex. Can. J. Research, C, 21 : 211–234. 1943.
20. Nobles, M. K. Studies in forest pathology. VI. Identification of cultures of wood-rotting fungi. Can. J. Research, C, 26 : 281–431. 1948.
21. Peck, C. H. N.Y. State Museum Rept. 43 : 69. 1890.
22. Peck, C. H. N.Y. State Museum Rept. 47 : 145. 1894.
23. Rishbeth, J. Observations on the biology of *Fomes annosus*, with particular reference to East Anglian pine plantations. 1. The outbreaks of disease and ecological status of the fungus. Ann. Botany (n.s.), 14 : 365–383. 1950.
24. Rogers, D. P. and Jackson, H. S. Notes on the synonymy of some North American Thelephoraceae and other resupinates. Farlowia, 1 : 263–328. 1943.
25. Saccardo, P. A. and Sydow, P. Sylloge Fungorum, 16 : 194. 1902.
26. Vandendries, R. Nouvelles recherches sur la sexualité des Basidiomycètes. Bull. soc. roy. botan. Belg. 56 : 1–25. 1923.
27. Vandendries, R. and Robyn, G. Nouvelles recherches expérimentales sur le comportement sexuel de *Coprinus micaceus*. II. Acad. roy. Belg. Classe Sci. Mém. 4 : 3–117. 1929.

CONTENTS OF VOLUME 30